

**STUDIES ON THE  
COMPOSITION OF THE  
RUMINAL MICROBIAL  
COMMUNITY USING  
GRASS SILAGE AND CORN  
SILAGE**



Melanie Lengowski







Institute of Animal Science  
University of Hohenheim  
Prof. Dr. M. Rodehutschord



# **STUDIES ON THE COMPOSITION OF THE RUMINAL MICROBIAL COMMUNITY USING GRASS SILAGE AND CORN SILAGE**

DISSERTATION

submitted in fulfilment of the requirements for the degree  
Doktor der Agrarwissenschaften  
(Dr. sc. agr. in Agricultural Sciences)

to the  
Faculty of Agricultural Science

presented by  
Melanie Lengowski  
born in Leonberg, Germany

2016

Die vorliegende Arbeit wurde am 23.09.2016 von der Fakultät Agrarwissenschaften der Universität Hohenheim als „Dissertation zur Erlangung des Grades eines Doktors der Agrarwissenschaften“ angenommen.

Tag der mündlichen Prüfung:

30. Januar 2017

Leitung des Kolloquiums:

Prof. Dr. J. Wünsche

Berichterstatter, 1. Prüfer:

Prof. Dr. M. Rodehutschord

Berichterstatter, 2. Prüfer:

Prof. Dr. Q. Zebeli

3. Prüfer:

Jun. Prof. Dr. J. Seifert

This work was financially supported by the German Research Foundation (DFG) through grant number BO 2960/1-2





## TABLE OF CONTENTS

1	INTRODUCTION, BACKGROUND AND OBJECTIVE .....	1
2	GENERAL DISCUSSION.....	13
2.1	METHODICAL CONSIDERATIONS.....	15
2.1.1	EXPERIMENTAL DESIGN AND <i>IN VITRO</i> INCUBATION .....	15
2.1.2	ANALYSIS OF MICROBIAL POPULATIONS.....	20
2.2	INFLUENCE OF SILAGE ON RUMINAL MICROBIAL COMMUNITY .....	22
2.3	FERMENTATION CHARACTERISTICS AND EFFICIENCY OF MICROBIAL CRUDE PROTEIN SYNTHESIS IN THE RUSITEC EXPERIMENTS .....	29
2.4	DIURNAL CHANGES.....	32
2.5	CONCLUSIONS AND PERSPECTIVES.....	35
	REFERENCES.....	39
3	INCLUDED MANUSCRIPTS.....	51
4	SUMMARY .....	116
5	ZUSAMMENFASSUNG .....	123



**LIST OF TABLES**

with the exception of tables presented in Manuscript 1-3

**TABLE 1:** 16S rRNA, mcrA and 18S rRNA gene copy numbers of total bacteria, methanogens, and protozoa, and 16S rRNA gene copy numbers of *Fibrobacter succinogenes*, *Ruminococcus albus*, *Prevotella bryantii*, *Clostridium aminophilum*, and *Selenomonas ruminantium* during incubation of grass silage or unsupplemented corn silage in period 2 of Experiment 1 and Experiment 2. .... 17

**TABLE 2:** Spectrophotometrically measurements (NanoDrop-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA) of DNA purity and concentration in DNA extracts obtained by different extraction protocols (gray shaded areas indicate the extraction protocols that were used in the current thesis)..... 21



## LIST OF FIGURES

With the exception of figures in Manuscript 1-3

- FIGURE 1:** Proportion of *Fibrobacter succinogenes*, *Clostridium aminophilum*, *Prevotella bryantii*, *Ruminococcus albus*, and *Selenomonas ruminantium* in fermenter liquids and feed residues at different times of incubation in the second period of **Experiment 1** (as percentage of total bacterial 16S rRNA gene coy numbers; mean;  $n \geq 2$ ;  $*P \leq 0.05$ ;  $***P \leq 0.001$ ) ..... 33
- FIGURE 2:** Proportion of *Fibrobacter succinogenes*, *Prevotella bryantii*, *Ruminococcus albus*, *Selenomonas ruminantium*, *Ruminobacter aminophilus*, and *Ruminococcus flavefaciens* in liquid and solid fraction at different sampling times in **Experiment 3** (as percentage of total bacterial 16S rRNA gene coy numbers; mean;  $n \geq 2$ ;  $*P \leq 0.05$ ;  $**P \leq 0.01$ ;  $***P \leq 0.001$ ) ..... 34



## LIST OF ABBREVIATIONS

with the exception of abbreviations only used in Manuscript 1-3

ADF	acid detergent fiber
CP	crude protein
CS	corn silage
DNA	deoxyribonucleic acid
e.g.	exempli gratia
Exp.	experiment
g	gram
GS	grass silage
h	hours
HAB	hyper ammonia producing bacteria
min	minutes
ml	milliliter
NDF	neutral detergent fiber
n.s.	not significant
PCR	polymerase chain reaction





# CHAPTER 1

## INTRODUCTION, BACKGROUND AND OBJECTIVE



## **1 INTRODUCTION, BACKGROUND AND OBJECTIVE**

The rumen is a complex ecosystem that hosts a diverse microbial community mainly comprising of anaerobic bacteria, methanogens, protozoa, and fungi. These microorganisms break down feed constituents ingested by the host animals while primarily producing volatile fatty acids, microbial biomass, and gases. The volatile fatty acids are absorbed through the rumen epithelium and serve as the main energy source for the host animal. In general, the ruminal microbial community and the host animal are two separate organisms that live in a symbiotic relationship. The host animal supplies a relatively constant environment, by providing high quantities of nutrients through continuous feed intake, regulated temperatures (38-41 °C), a large amount of saliva which is a bicarbonate-phosphate buffer with a pH of 8 for buffering the volatile fatty acids produced during microbial fermentation, and the removal of soluble inhibitory end products of digestion as well as undigested particulate matter (Mackie et al., 2001). Furthermore, the host animal provides urea through saliva, formed from the ammonia absorbed through the rumen wall, which is then converted back into ammonia by the rumen organisms and again available for microbial synthesis. Urea is also secreted through the rumen wall and added to the recycled ammonia pool. This environment favors the microorganisms and they break down the feed constituents fed by the host animal.

The rations fed to ruminants of agricultural importance, such as cows and sheep, in Europe, usually contains grass silage (GS), corn silage (CS), hay, concentrates, and minerals. The ruminal microbial community provides enzymes which are necessary to digest plant polymers (starch, cellulose, hemicellulose, pectins, and proteins). Thus, the ruminants are allowed to efficiently utilize the energy and nutrients contained in fibrous materials that cannot be used by monogastric animals. The microorganisms also synthesize vitamins B and K, and detoxify phytotoxins and mycotoxins (Mackie et al., 2001). Furthermore, the microorganisms produce microbial proteins which are digested by the host animal in the small intestine, to obtain energy and amino acids. In case of poor quality dietary protein and non-protein nitrogen, microbial protein contains a favorable amino acid composition and leads to better protein quality for the host animal.

The ruminal microbial community has been under investigation since the first half of the 1900s, and the rumen belongs among the most extensively and intensively investigated anaerobic ecosystems. Nonetheless, relatively little is known about the species and numbers of organisms as well as their activities and the interactions between them, due to the high diversity and variability of rumen microorganisms.

### IDENTIFICATION AND QUANTIFICATION OF MICROORGANISMS

In the early days of rumen microbiology, microorganisms were investigated by cultivation-based techniques, usually in pure cultures. This involves the isolation of a single strain of bacteria,

archaea, protozoa, or fungi, and its subsequent characterization. At the end, it provides detailed information on one organism. In the rumen, however, a wide range of microorganisms occurs and interacts among each other. Hence, information obtained from pure cultures is limited when it comes to interpretation of total rumen activity. Furthermore, the microorganisms in the rumen are predominantly strict anaerobes. Anaerobic cultivation methods are difficult and time-consuming. Moreover, it is now clear that only a very small fraction of the microbial diversity in natural ecosystems has been recovered by cultivation (Amann et al., 1995). In this regard, the development of more sensitive and accurate molecular detection methods has brought forth new opportunities. Methods for direct retrieval and sequence analysis of some target genes, mainly those of ribosomal (r) RNA, provide the possibility of determining the genetic diversity and phylogenetic relationship of microorganisms in different ecosystems, without cultivation. Presently, the most useful and most investigated phylogenetic marker molecules are the rRNA, especially 16S rRNA, and to a lesser extent 23S rRNA (Ludwig and Schleifer 1994) in prokaryotes and 18S rRNA in eukaryotes. These RNA genes occur in all cells and have elements which are invariant in sequence, size, or structure in molecules of phylogenetically related organisms or groups, but are different in others. These different highly variable regions can be used for identification of the organism or the group it belongs to (Ludwig and Schleifer 1994; Woese 1987). Analyses of target genes amplified directly from the rumen-extracted DNA, using polymerase chain reaction (PCR) techniques, may help describe the ruminal microbial community. In a conventional PCR, the amplified product is detected at the end of the analysis by running the DNA on an agarose gel. In contrast, real-time quantitative (q) PCR allows quantification of amplified DNA in real time, during the exponential phase of the reaction. This is made possible by including a fluorescent molecule in the reaction that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. With the real-time qPCR, an absolute (with the use of standard curves) or relative quantification of different microorganisms can be measured in a short time, without extensive cultivation methods. The real-time qPCR is only one of several methods which have revolutionized microbiological research. Newer research perspectives through these molecular techniques can lead to a more accurate description of the ruminal microbial community.

#### RUMINAL MICROBIAL COMMUNITY

Bacteria are highly predominant in the rumen (with  $10^{11}$  viable cells per gram rumen content comprising up to 1000 phylotypes), followed by archaea ( $10^7$ - $10^9$  per gram), and a variety of ciliate protozoa ( $10^4$ - $10^6$  per gram distributed over 25 genera; Mackie et al., 2001). Anaerobic fungi are also present ( $10^2$ - $10^4$  per gram; Mackie et al., 2001). The microorganisms in the rumen hydrolyze the plant celluloses, hemicelluloses, pectins, fructosans, starches, and other polysaccharides to monomeric or dimeric sugars, which are fermented, along with simple sugars

in the vegetation, to yield various products, some of which may be subject to further microbial action (Hobson, 1997). Proteins are hydrolyzed to amino acids and peptides. The amino acids are then deaminated to ammonia and fatty acids. The bulk of ammonia is absorbed through the rumen wall (Wallace et al., 1997) to be converted into urea, while some of the ammonia is used by the microorganisms themselves for microbial growth. The final products of the microbial actions produced by consortia of bacteria, protozoa, fungi, and archaea, are acetic, propionic and butyric acids, methane, and carbon dioxide (Hobson, 1997). Some of the carbon dioxide, ammonia, and other products of microbial metabolism along with some feed components, provide the material for microbial cell synthesis. The microbial cell proteins form the animal's feed protein which is digested in the abomasum and large intestine (Hobson, 1997).

The rumen bacterial community comprises a very large number of species, which are either substrate specific or generalists. For example, *Ruminobacter amylophilus*, that utilizes only starch or its degradation products, or *Fibrobacter succinogenes*, which ferments cellulose, are substrate specific. The generalists e.g. *Butyrivibrio fibrisolvens* may hydrolyze a broad range of substrates like starch, cellulose, xylan, and pectin (Stewart et al., 1997). Furthermore, the rumen hosts some species like *Selenomonas ruminantium*, which show limited ability to hydrolyze polymers but which use a wide range of hydrolysis products generated by other ruminal microorganisms.

The domain archaea is represented by the methanogens in the rumen (Mackie et al., 2001). The methanogenic archaea in the rumen includes a wide range of different species. Most species can grow on hydrogen and formate as their energy sources, and use the electrons derived from hydrogen (or formate) to reduce carbon dioxide to methane. The ability to use formate is based on their ability to convert formate to hydrogen and carbon dioxide and the subsequent reduction of carbon dioxide to methane. Certain species of methanogenic archaea use acetate, methanol, and mono-, di- and trimethylamines, but hydrogen and carbon dioxide are the principal substrates for the methane production in the rumen (Janssen and Kirs 2008; Wolin et al., 1997). Although methanogens make up only a small part of the rumen microbial biomass, they play an important role in rumen function and animal nutrition. Efficient hydrogen removal leads to a more nutritionally favorable pattern of volatile fatty acid formation and to an increased rate of fermentation, due to elimination of the inhibitory effect of hydrogen on microbial fermentation.

The rumen protozoa are mostly ciliates. Despite the metabolic diversity of the ciliate protozoa and their large biomass in the rumen, protozoa are not essential for the survival and development of the host animal. Nevertheless, they directly affect the wellbeing, productivity, and environmental impact of the host animal (Williams and Coleman 1997). The most important role of rumen protozoa is their ability to engulf large molecules, proteins, and carbohydrates. Protozoa show proteolytic activity and play a major role in protein degradation. They hydrolyze particulate proteins of an appropriate particle size (Wallace et al., 1997) and ruminal bacteria, which are their main source of protein. Thus, protozoa have a regulatory effect on bacterial nitrogen turnover in

the rumen (Bach et al., 2005). Furthermore, the uptake, killing, and digestion of bacteria by rumen ciliates and release of the digestion products in the rumen medium is a very important part of nitrogen cycling, because the amino acids released are metabolized for growth by the remaining bacteria (Williams and Coleman, 1997). Although amylolytic protozoa are not essential in starch utilization, it has been suggested that engulfing starch granules limits the amount of starch available for rapid fermentation by bacteria, and helps decrease the risk of acidosis (Mackie et al., 1978).

Anaerobic fungi are involved in carbohydrate degradation (Mackie et al., 2001). They produce a wide range of enzymes that can hydrolyze the major structural carbohydrates of plant cell walls as well as a number of polysaccharides (Orpin and Joblin, 1997; Trinci et al., 1994). They are described to colonize and degrade lignified plant cell walls which are not extensively colonized by other ruminal microorganisms (Trinci et al., 1994). As a result, anaerobic fungi supply the bacteria with access to the cellulose (Castillo-González, et al., 2014) and, thus, assist cellulose fermentation.

Based on the specifications of its diverse members, the ruminal microbial community is highly responsive to changes in feeding patterns of the host animal.

#### INFLUENCE OF DIFFERENT RATIONS ON THE RUMINAL MICROBIAL COMMUNITY

A change in ration creates a disturbance in the ruminal ecosystem (Monteils et al., 2012). This disturbance caused is primarily associated with environmental changes for the ruminal microbial community (e.g. nutrient availability, pH). While high proportions of silages promote cellulolytic bacteria, more concentrate in the ration promotes amylolytic bacteria. The energy requirement of high-performance dairy cows results in a high proportion of concentrate being used in the ration. This leads to low pH due to fast formation of volatile fatty acids by the microorganisms and reduced rumination due to the lower structural fiber content. The low pH may induce rumen acidosis, which has negative effects on the host animal and its performance (Dijkstra et al., 2012). There are many studies which have investigated the effects of different rations and feed additives on fermentation characteristics (Abrahamse et al., 2008; Brask et al., 2013; Saro et al., 2014; Valdez et al., 1977; Whelan et al., 2013) as well as on the performance of the cows (Boguhn et al., 2010; Dewhurst et al., 2001; Dewhurst 2013; Hassanat et al., 2013; O'Mara et al., 1998; Zhu et al., 2013), and on the microbial community (Owens et al., 2009; Petri et al., 2012; Staerfl et al., 2012; Tajima et al., 2001; Wang et al., 2009). The effect of silage on the ruminal microbial community has also been shown (Belanche et al., 2013; Huws et al., 2010; Kong et al., 2010; Martínez et al., 2010c). In Europe, GS and CS are two of the most important silages used in rations for dairy cows. While GS contains relatively high amounts of crude protein (CP) and fiber fractions which promote the proteolytic and cellulolytic bacteria, CS has a higher content of soluble carbohydrates which promotes the amylolytic species in the rumen. Thus, different

fermentation end products are produced (Brask et al., 2013; Doreau et al., 2011; Huws et al., 2010). During the fermentation of cellulose, the volatile fatty acid produced most is acetate; while fermentation of starch and highly soluble carbohydrates, result in more propionate and butyrate and, thus, in a lower acetate to propionate ratio. The CP in GS can be more rapidly degraded by the ruminal microbial community, than CP from CS (Givens and Rulquin, 2004). But, CS provides a higher amount of highly soluble carbohydrates for the microorganisms, which can result in higher efficiency of microbial CP synthesis *in vivo*, as compared to GS (Givens and Rulquin, 2004), since a more synchronized supply of energy and protein is possible. Consequently, GS and CS have diverse effects on the ruminal microbial community.

There are different possibilities for investigating the effect of diverse rations on the fermentation characteristics and the microbial community in ruminants, one of which is through *in vivo* studies. Since these studies are expensive and time-consuming, a second possibility is the performance of *in vitro* studies. Over the years, a number of different *in vitro* systems have been developed to estimate digestibility and extent of ruminal degradation of feeds, as well as to study variations in the ruminal microbial community in response to changes in feeding conditions (López, 2005). *In vitro* systems have been used for feed evaluation, to investigate mechanisms of microbial fermentation, and to study the mode of action of anti-nutritive factors, additives, and feed supplements (López, 2005).

#### IN VITRO SYSTEMS

*In vitro* systems represent biological models that simulate the *in vivo* digestion process with different levels of complexity (López, 2005). These systems allow the realization of a large number of treatments in sufficient replication within a relatively short period. In addition, it also permits the testing of higher, in some cases, potentially toxic to the host animal, levels of feed additives along with lower experimental costs as compared to an animal trial (Hristov et al., 2012). Besides, employing *in vitro* systems allows the establishment of well-controlled environmental testing conditions, without the inherent variability that occurs when using individual animals (Czerkawski and Breckenridge, 1977). Furthermore, *in vitro* studies allow manipulation of properties, so as to study responses when one factor is varied and controlled without the interaction of other related factors (López, 2005). Moreover, with *in vitro* studies, it is possible to follow the metabolic response to one single feed by avoiding the influence exerted by another feed and, therefore, to describe its own characteristic value (Gizzi et al., 1998). Thus, *in vitro* studies may be used to study individual processes for providing information about their nature and sensitivity to various factors (López, 2005).

The disadvantages of *in vitro* systems are the absence of mastication, of interaction with the rumen wall, and the lack of recycling of nitrogen via rumino-hepatic circulation. Furthermore, the

passage rate of different feed particles varies in the rumen but remains the same for all feed stuffs in *in vitro* systems.

In addition to simple *in vitro* systems like batch cultures, there are also more complex systems. Two such systems have to be distinguished: the semi-continuous Rumen simulation technique (Rusitec; Czerkawski and Breckenridge, 1977) and the continuous rumen simulation system (Hannah et al., 1986; Hoover et al., 1976). The latter system has the advantage of supplying feed continuously in fermenter liquids which simulate the native feed ingestion during the day. The removal of liquid and solid effluent in these systems is difficult. In the system from Hoover et al. (1976) the mixed fluid and solid media flow out at one overflow port, while in the dual flow continuous culture system from Hannah et al. (1986), fermenters are equipped with an overflow port, through which both liquid and solid fraction passes, and a second filtered output assists in removal of primarily liquid media.

The installation of semi continuous systems is easier as compared with continuous systems. In these systems, the infusion of buffer solution into the fermenter and the removal of liquid effluent by overflowing are continuous. However, feed input and removal of solids occur once daily.

For this doctoral thesis, the Rusitec (Czerkawski and Breckenridge, 1977) has been used for *in vitro* experiments. This system has six reaction vessels with a capacity of 800 ml each in a warm water bath. Inside each fermenter, a perforated feed container is placed which contains 2 nylon bags to incubate feed. At the beginning of the experiments, fermenters are filled with a mixture of rumen liquids and artificial saliva (McDougall, 1948). Afterwards, the mixture of feed and fermenter liquid is ensured by vertical movement of the feed containers. Artificial saliva is continuously infused and the effluent is collected in glass bottles standing inside a cooled water bath to stop microbial fermentation. Gas production is measured in plastic bags attached to the vessel.

#### RUSITEC EXPERIMENTS

The Rusitec was used in a lot of studies to investigate the effect of different feedstuffs (Lee et al., 2011; Witzig et al., 2015; Zhao et al., 2013), rations (Boguhn et al., 2008; Witzig et al., 2010a; Witzig et al., 2010b) and feed additives (Klevenhusen et al., 2015; Narvaez et al., 2013; Seeling et al., 2006; Wischer et al., 2013) on the ruminal microbial community.

While assessing studies which investigate the effect of silage on rumen microbial community (Ghaffari et al., 2015; Witzig et al., 2010a; Witzig et al., 2010b) it was found that in most studies silage was combined with concentrate and only in a few, was silage incubated alone (Belanche et al., 2013; Witzig et al., 2015). Therefore, in the majority of cases it is not possible to evaluate the effect of only silage on the microbial community.

A major problem with Rusitec studies carried out in the last few years is the high variation in sampling location for microbial analyses. In some studies, samples were taken from the liquid



effluent (Boguhn et al., 2008; Narvaez et al., 2013; Witzig et al., 2010a; Witzig et al., 2010b), while in others, samples were taken from the fermenter liquid (Belanche et al., 2013; Russi et al., 2002; Witzig et al., 2015; Zhao et al., 2013; Ziemer et al., 2000). Only in a few studies, were samples also collected from the feed residues (Boguhn et al., 2008; Narvaez et al., 2013; Witzig et al., 2015; Zhao et al., 2013). Since most ruminal bacteria are associated with undigested feed particles (Olubobokun et al., 1988), examination of only the fermenter or effluent liquids do not provide a complete evaluation of the microbial community. Furthermore, due to the different sampling locations, comparisons between the studies are difficult.

An additional problem is the sampling time. In most studies, samples were collected at one time point (Narvaez et al., 2013; Russi et al., 2002; Witzig et al., 2010a; Witzig et al., 2010b; Witzig et al., 2015). Meanwhile, Bryant and Robinson (1968) already showed, in 1968, significant variations between sampling times in heifers. In *in vitro* as well as in *in vivo* studies, experiments where samples are taken over time are rare (Saro et al., 2014; Zeitz et al., 2013; Ziemer et al., 2000). Furthermore, despite the importance of a functional microbial community in the Rusitec, the adaption of the ruminal microbial community to this *in vitro* system was only investigated in one study (Prevot et al., 1994). A further study examines the fermentation characteristics during the adaption phase (Martínez et al., 2011).

In the last few years, some authors (Boguhn et al., 2006; Hildebrand et al., 2011a; Hildebrand et al., 2011b) have investigated the effect of a different CS to GS ratio on the ruminal microbial CP synthesis and the fermentation characteristics in the Rusitec. The degradation of organic matter, fiber fractions, and non-structural carbohydrates, as well as microbial nitrogen flow, was higher for GS than for CS (Hildebrand et al., 2011a; Hildebrand et al., 2011b). Boguhn et al. (2006) observed that the CP content in the rations was the only dietary chemical fraction that had significant effect on the microbial CP synthesis. Furthermore, the inclusion rate of CS in the ration also tended to improve the efficiency. Nonetheless, a large part of the variation in the efficiency of microbial activity still remains unexplained. Hildebrand et al. (2011a) investigated the effect of different CS to GS ratios, with a constant proportion of soybean meal, on the efficiency of microbial CP synthesis in the Rusitec. In contrast to Givens and Rulquin (2004), who reported a higher efficiency of ruminal CP synthesis with CS compared to GS *in vivo*, in this *in vitro* study a higher efficiency with higher proportions of GS was found. One reason for this finding could be an improved efficiency of microbial CP synthesis by a higher degradation of organic matter from GS and thus, increased availability of nitrogen. In a follow-up study, Hildebrand et al. (2011c) supplemented urea to CS to balance the higher nitrogen content in GS as compared to CS. The urea supplement increased the efficiency of microbial CP synthesis for CS, but not to the level found in the case of GS. This might be due to the fact that different microorganisms prefer different sources of nitrogen. Griswold et al. (1996) and Carro and Miller (1999) showed that different nitrogen forms affect the bacterial efficiency in continuous systems.

Argyle and Baldwin (1989) reported that the addition of amino acids and/or peptides in the rumen stimulated microbial growth. In contrast, Soto et al. (1994) found no influence of peptides, amino acids, or urea on the numbers of total bacteria, cellulolytic bacteria, and ciliate protozoa in grass hay fed sheep. Only in pure cultures, Soto et al. (1994) reported a stimulating effect of peptides and amino acids on the growth of *Ruminococcus albus*, *R. flavefaciens*, and *F. succinogenes*. Similar results in pure culture were observed by Atasoglu et al. (1998) who reported the increase in the apparent growth yields by between 8% and 57%, depending on the species (*Prevotella bryantii*, *S. ruminantium*, *Streptococcus bovis*), when 1 g of peptides or amino acids were added to the medium. Ha et al. (1986) investigated the effect of urea and protein sources on the microbial CP synthesis in steers, and found a higher efficiency of microbial CP synthesis in steers fed on urea as compared to protein sources. But, Chikunya et al. (1996) reported the effect of nitrogen source on the numbers of total viable bacteria in sheep, when the energy source is fermented rapidly. In this study, grass hay or molassed sugar beet pulp was used (Chikunya et al., 1996).

There are also studies which investigated the effect of GS and CS on the ruminal microbial community in the Rusitec (Witzig et al., 2010a; Witzig et al., 2010b; Witzig et al., 2015). These studies observed the effect of GS and CS on the ruminal *Bacteroides-Prevotella* (Witzig et al., 2010a) and *Firmicutes* community (Witzig et al., 2010b). However, the silage was not incubated alone but combined with soybean meal, and samples were collected at one time point at the end of the Rusitec run, from the fermenter effluent. Witzig et al. (2015) incubated only CS with urea or GS without concentrate and also found an effect of silage on the ruminal microbial community in fermenter liquids and feed residues in the Rusitec.

#### OBJECTIVES OF THE THESIS

On the basis of the currently available literature, the aim of this doctoral thesis was to improve the knowledge about the effects of GS and CS on the ruminal microbial community *in vitro* and *in vivo*, since, most studies which investigated the effect of GS and CS on the ruminal microbial community, until now, have been *in vitro*. Only Staerfl et al. (2012) have observed these effects *in vivo*. Another aim of this doctoral thesis was to examine the behavior of the ruminal microbial community during its adaption to the Rusitec system. Three manuscripts, which investigated the changes in rumen microbial community during adaption to the Rusitec as well as diurnal changes *in vitro* and *in vivo*, were provided. Furthermore, due to the contradictory results reported about the effect of GS and CS on the efficiency of ruminal microbial CP synthesis *in vivo* and *in vitro*, the effect of different nitrogen sources on the rumen microbial community and fermentation characteristics *in vitro* were examined.

**MANUSCRIPT 1: Changes in ruminal microbial community composition during adaption to an *in vitro* system and the impact of different forages.**

Despite the great number of published studies, no information is available on the behavior of ruminal microbial community during the adaption to the Rusitec system. Therefore, the objective of the first study was to determine the changes in composition of the ruminal microbial community composition within the first 48 hours of adaption to the Rusitec. In order to investigate the effect of silage, no concentrate was used in this study. Due to different nutrient levels, changes in ruminal microbial populations can be expected during the day. Thus, the second objective was to investigate the diurnal changes of the ruminal microbial community after adaption to the Rusitec system.

The manuscript was published in *PLOS one*.

**MANUSCRIPT 2: Effects of supplementing maize silage with different nitrogen sources on ruminal fermentation and microbial populations *in vitro*.**

The efficiency of the microbial CP synthesis of ruminal microorganisms depends on the feed fed to the animal. Former studies reported that differences in the efficiency of microbial CP synthesis depended on the availability of nitrogen. Furthermore, different species prefer different nitrogen sources. Thus, the second experiment of the doctoral thesis was conducted to evaluate the effects of different nitrogen sources added to CS on the microbial populations and the efficiency of microbial protein synthesis in the Rusitec system.

The manuscript was submitted to *PLOS one*.

**MANUSCRIPT 3: Effects of corn silage and grass silage in ruminal rations on diurnal changes of microbial populations in the rumen of dairy cows.**

Current understanding of the effects of CS and GS on within-day changes of ruminal microbial populations stem solely from the first *in vitro* experiment, demonstrating that sampling time has a major effect on the results for different species. Thus, it is likely that both silages as well as the sampling time have a significant effect on the composition of the ruminal microbial community *in vivo*. Therefore, the third experiment was conducted to evaluate diurnal changes of ruminal microbial populations in dairy cows fed with CS- or GS-based mixed rations.

The manuscript was published in *Anaerobe*.



## CHAPTER 2

### GENERAL DISCUSSION



## 2 GENERAL DISCUSSION

In Europe, GS and CS are important silages for the feeding of dairy cows and fattening bulls. Due to the different nutrient composition, they influence the microbial community in different ways. However, in most studies, silage was combined with concentrate; so, it is not possible to evaluate the effect of the single silage on the ruminal microbial community. Based on findings from studies of the present thesis, the effect of GS and CS on the ruminal microbial community, the fermentation characteristics, as well as the efficiency of microbial CP synthesis is debated upon in this general discussion. The discussion about the efficiency of microbial CP synthesis is limited to the two Rusitec experiments (Exp. 1 and 2) because in the *in vivo* experiment (Exp. 3), the efficiency of microbial CP synthesis was not investigated. The last part will deal with the effect of different sampling times on the ruminal microbial community.

### 2.1 METHODOICAL CONSIDERATIONS

The experimental design as well as the analysis of microbial population in this thesis comprises some methodical aspects which can be of relevance for the results of the present work. These will be discussed first.

#### 2.1.1 EXPERIMENTAL DESIGN AND *IN VITRO* INCUBATION

##### RUSITEC

The Rusitec is an *in vitro* system designed to simulate the rumen and its fermentation procedure. For this purpose, the strict anaerobic ruminal microbial community has to be transported from the donor animal into the Rusitec system. During this process, the injection of oxygen is hardly avoidable.

In the current thesis, the inoculum for the Rusitec was obtained from three (Exp. 1) and two (Exp. 2) lactating cows (Jersey) fitted with permanent cannulas. The liquid rumen contents were filled in pre-warm thermos flasks, while solid rumen contents were filled in little plastic boxes. Both containers were filled to the top and immediately closed to prevent air from entering, thus minimizing the oxygen influence. The plastic boxes filled with solid rumen contents were placed in a polystyrene box together with hot-water bottles and subsequently transported together with the thermos flasks into the laboratory. The liquid rumen contents of all three cows were mixed and filtered through two layers of linen cloth before the fermenters were filled. In order to avoid oxygen injection during this filling process, an overflow with carbon dioxide or nitrogen over the fermenters is necessary. But the use of carbon dioxide and nitrogen in the laboratory requires special precautions to avoid an accumulation of these gases in the breathing air. This was not possible in this doctoral project. Nevertheless, in order to avoid high oxygen injection, the fermenters were filled as fast as possible. In a few Rusitec studies (Cieslak et al., 2014; Narvaez et al., 2013; Zhao et al., 2013), an overflow with carbon dioxide has been used, while in others, it

was not. In these studies, either only 30 minutes were spent between the collection of rumen contents and filling of the fermenters (Carro and Miller, 1999; Martínez et al., 2010a), or no details were given (Lee et al., 2011; Russi et al., 2002; Witzig et al., 2015).

Thus, it could be possible that during this doctoral project, more oxygen was injected unlike in the other studies. One indication for this problem could be the lower degradation characteristics in Exp. 1 and 2 as compared to other Rusitec studies. In both experiments (Exp. 1 and 2), the degradation of organic matter was between 40% and 47%. In other Rusitec studies, the digestibility of organic matter was higher (Cieslak et al., 2014; Lee et al., 2011; Martínez et al., 2010a; Zhao et al., 2013); e.g. Cieslak et al. (2014) reported an organic matter digestibility between 51% and 54%. The fiber degradation in the literature was also higher (Carro and Miller, 1999; Martínez et al., 2010a; Zhao et al., 2013). The neutral detergent fiber (NDF) degradation in the current thesis (Exp. 1 and 2) was between 8% and 19% while Zhao et al. (2013) reported a NDF disappearance between 21% and 27%. Of course, the degradation of organic matter or fiber depends on the substrate which was incubated. But all studies mentioned above used grass hay, alfalfa hay, and silage, GS and/or CS, while in the current thesis GS and CS were used.

Another possible reason for the lower fiber degradation in Exp. 1 and 2 as compared to previous studies could be the use of isoacids. Carro and Miller (1999) and Russi et al. (2002) additionally mixed isoacids into the buffer since these constitute essential growth factors for cellulolytic bacteria (Dehority, 2003) and are especially important when the diet contains large proportions of non-protein nitrogen. But results from Exp. 2 showed highest acid detergent fiber (ADF) degradation among CS treatments for urea supplementation. For CS supplemented with urea, a similar level of isoacid production was observed like for CS alone. Thus, the availability of isoacids under the given conditions did not enhance the fiber degradation and it seems possible that similar results could be observed if isoacids were supplemented in Exp. 1. But, it cannot be excluded that availability of isoacids during the adaption phase could have had a positive effect on the establishment of cellulolytic microorganisms.

A further problem of the Rusitec system is the feed input and removal of feed residues. For changing the feed bags, the feed container inside the fermenter has to be taken out by manually opening the fermenter cover. The “old” feed bag incubated for the last 48 h is removed and the fresh feed bag is placed in the feed container. This procedure leads to an entry of oxygen in the otherwise strict anaerobic system. Thus, in Exp. 1 and 2, an initial (during filling the fermenters) and repeated (during changing the feed bags, and sampling of liquid fraction) oxygen input in the system occurs, which could have had an effect on the ruminal microbial community. In particular, the cellulolytic bacteria and methanogens, which are very sensitive to oxygen, may be reduced by this procedure. In Exp. 2, sampling of fermenter liquids were developed, since a little sealable hole was drilled in the fermenter lid and samples were taken with a bulb pipette. Thereby, the



oxygen input was minimized as compared to Exp. 1, which had a positive effect on the abundance of most of the species as shown in Table 1. But there could also be other possibilities which can have an influence of the microbial community. In Exp. 1, repeated sampling occurred during the adaption phase during which the fermenter lid was opened. This might have affected the establishment of different microbial species within the fermenters. Furthermore, the different donor animals could have an influence, as shown by Boguhn et al. (2012). Indeed, in both experiments, lactating Jersey cows were used and fed with total mixed rations contained CS and GS for *ad libitum* consumption. However, the identical animals were not used. The silages that were incubated in the Rusitec could also have an influence. Silages used in both Rusitec were not the same but chemical compositions were nearly identical for both GS and CS.

Nevertheless, Martínez et al. (2010a) reported that the Rusitec simulated *in vivo* fermentation very closely during incubation of high-forage diets. Furthermore, Czerkawski and Breckenridge (1977) showed similar types and quantities of fermentation products *in vitro*, as those generated in the animals used as donor animals.

TABLE 1: 16S rRNA, mcrA and 18S rRNA gene copy numbers of total bacteria, methanogens, and protozoa, and 16S rRNA gene copy numbers of *Fibrobacter succinogenes*, *Ruminococcus albus*, *Prevotella bryantii*, *Clostridium aminophilum*, and *Selenomonas ruminantium* during incubation of grass silage or unsupplemented corn silage in period 2 of Experiment 1 and Experiment 2.

Microbial groups/species	Forage source	Liquid fraction				Solid fraction			
		Exp <sup>1</sup> . 1	(SD)	Exp. 2	(SD)	Exp. 1	(SD)	Exp. 2	(SD)
<b>Bacteria</b>	CS <sup>2</sup>	25.5	(14.1)	15.6	(2.04)	330	(139)	215	(35.0)
	GS <sup>3</sup>	26.3	(13.2)	11.2	(1.42)	281	(125)	211	(31.3)
<b>Methanogens</b>	CS	0.10	(0.083)	0.16	(0.028)	0.25	(0.19)	0.30	(0.031)
	GS	0.34	(0.41)	0.28	(0.043)	0.096	(0.046)	0.50	(0.066)
<b>Protozoa</b>	CS	0.14	(0.074)	0.40	(0.088)	0.03	(0.033)	0.74	(0.02)
	GS	0.01	(0.010)	0.018	(0.004)	0.004	(0.004)	0.022	(0.006)
<b><i>F. succinogenes</i></b>	CS	0.11	(0.057)	0.45	(0.066)	4.42	(2.10)	15.3	(4.71)
	GS	0.021	(0.015)	0.54	(0.073)	0.40	(0.22)	5.31	(1.45)
<b><i>R. albus</i></b>	CS	0.006	(0.004)	1.39	(0.22)	0.33	(0.22)	4.89	(1.41)
	GS	0.016	(0.012)	2.15	(0.27)	0.85	(0.78)	6.05	(1.86)
<b><i>P. bryantii</i></b>	CS	0.086	(0.043)	0.36	(0.13)	1.69	(0.89)	1.37	(0.74)
	GS	0.18	(0.091)	1.77	(0.61)	8.98	(13.2)	3.49	(1.93)
<b><i>C. aminophilum</i></b>	CS	0.053	(0.34)	0.13	(0.024)	0.37	(0.097)	0.87	(0.29)
	GS	0.075	(0.032)	0.032	(0.061)	0.93	(0.38)	0.40	(0.10)
<b><i>S. ruminantium</i></b>	CS	0.82	(0.49)	1.23	(0.26)	53.6	(21.0)	24.4	(6.16)
	GS	0.49	(0.31)	0.70	(0.15)	15.5	(7.30)	10.6	(2.57)

Data were detected by real-time quantitative PCR and expressed in copy number x 10<sup>8</sup> per mL liquid fraction or g solid fraction; Results of Experiment 1 were averaged over the 5 sampling times in period 2; mean (SD); n<sup>2</sup> ≥ 3; <sup>1</sup>experiment, <sup>2</sup>corn silage, <sup>3</sup>grass silage.

In the Rusitec, sampling of solid fraction is only possible during the change of feed bags due to the technical conditions of this *in vitro* system. Thus, the smallest possible interval between two

sampling times is 24 h. To get samples at this time point, the feed bag has to be opened and samples of feed have to be removed out of it. Following this, the feed bag is closed and placed back in the feed container and incubated again for further 24 h. This primarily has two disadvantages: First of all, oxygen is introduced to the system and into the feed bag. Thus, taking samples at shorter intervals means repeated opening of the fermenters and feed bags with unforeseeable consequences for the anaerobes due to more frequent oxygen introduction. Second, this bag cannot be used to determine the digestibility of nutrients since an indefinite amount of feed and solid associated microorganisms are taken out. Furthermore, removing feed from feed bags resulted in a lower substrate level for the next 24 h which also has an effect on the microbial fermentation. Thus, for repeated sampling from feed residues at shorter time intervals than 24 h, separate fermenters would be necessary, which are only operated until the respective sampling time. For this reasons, in the current thesis (Exp. 1 and 2), samples from the solid fraction were only taken at the end of each Rusitec run, from feed bags which were incubated at this time point for 24 h and 48 h, respectively. However, the interval of 24 h is relatively long. Craig et al. (1987a) showed that particle associated microbial matter in the rumen of dairy cows was highest an hour after feeding and lowest after 10 h. In this study, cows were fed at 12 h intervals. Furthermore, Edwards et al. (2008) investigated the initial stages of bacterial colonization of forage sources in dairy cows, and showed that bacterial numbers increased rapidly within 5 min and stabilized after 15 min of *in sacco* incubation. However, Koike et al. (2003) reported highest numbers of attached cells of cellulolytic bacteria after 24 h (*F. succinogenes* and *R. flavefaciens*) or 48 h (*R. albus*) of *in sacco* incubation of orchard grass hay in sheep. For all three species, a rapid colonization during the first 10 min was reported. In a later study, Koike et al. (2014) compared the colonization of untreated rice straw and sodium hydroxide-treated rice straw, and reported that the colonization patterns of rumen bacteria depend on the treatment of substrate. Furthermore, interactions between rumen bacteria occur, which have an influence on fiber digestion. Also Kozakai et al. (2007) reported the effect of mechanical processing on bacterial colonization of CS. The results demonstrated a more rapid attachment and heavier colonization of rumen bacteria to the mechanically processed CS as compared to the unprocessed CS. The authors took samples after 10 min, 2 h, 24 h, and 28 h of *in sacco* incubation. Depending on the silage (processed or unprocessed), highest numbers for *F. succinogenes* were found after 2 h or 48 h, respectively. Thus, it is necessary to consider at which time points the sampling of solid fraction is useful, depending on the substrate and aim of the respective study. In both Rusitec studies (Exp. 1 and 2) silages were ground through a 1 mm sieve, prior to incubation. Thus, it can be assumed based on the results of Kozakai et al. (2007) that colonization of feed particles was at a high level. Furthermore, Witzig et al. (2010a, 2010b) reported a higher relative abundance of *P. bryantii* and *R. albus* in the effluent of fermenters in which GS or CS were incubated ground to pass 1-mm screen, as compared to silages ground to pass 4-mm screen. But results of Exp. 3

indicated significant changes within 24 h, which suggest a shorter sampling interval than 24 h for samples from the solid fraction.

Sampling of fermenter liquid without possible introduction of oxygen in the Rusitec system is relatively simple. With the help of a little hole in the lid of the fermenter, it is possible to sample the fermenter liquid without opening the whole fermenter. The liquid can be obtained with a bulb pipette (Exp. 2). But the investigation of solid fraction is really important since 70 – 80% of rumen microbial matter is particle associated (Craig et al., 1987a). Thus, the liquid fraction alone cannot be representative for the total ruminal microbial community.

In the Rusitec system, it is necessary to use a bicarbonate buffer to simulate the saliva and maintain the pH in the fermenter at a constant level. The amount of buffer that flows into the fermenter during the day, and thereby the dilution rate, also has an effect on the fermentation characteristics, and especially on the protozoal community (Abe and Kumeno, 1973; Carro et al., 1995). In particulate, species with long generation intervals are negatively affected by the dilution rate as they could be simply washed out. Protozoa, which are known to have a long generation interval (Potter and Dehority, 1973), showed lower numbers *in vitro* as compared to *in vivo* (Martínez et al., 2010b; Ziemer et al., 2000). In the rumen of cows, the majority of protozoa (63 – 90%) are found either associated with feed particles or sequestered in the rumen wall (Hook, et al., 2012) and thus maintained in the rumen. But this is not possible in the Rusitec. In the current thesis, dilution rates were 590 ml (Exp. 1) and 612 ml (Exp. 2) per day which correspond to a dilution rate of 0.7 and 0.8 volume turnover per day, respectively. In both experiments (Exp. 1 and 2), decreasing numbers of protozoa were observed. Abe and Kumeno (1973) reported that the most adequate turnover rate for the maintenance of the entodiniomorph protozoa would be about 0.5 volumes per day. The authors showed that, at this turnover rate, the protozoa maintained their original numbers in a semipermeable culture system.

The loss of protozoal population in the Rusitec could also be a reason for the lower fiber degradation in the current studies (Exp. 1 and 2), since defaunation of ruminants significantly decrease rumen organic matter digestibility and, particularly, NDF and ADF digestibility (Newbold et al., 2015).

#### ADAPTION OF MICROBIAL COMMUNITY TO THE RUSITEC SYSTEM

Until now, only one study has investigated the behavior of ruminal microbial community during the adaption phase in the Rusitec system (Prevot et al., 1994). The authors identified and counted the bacterial species by immunofluorescence. Another study investigated the development of fermentation characteristics during the first days of a Rusitec run (Martínez et al., 2011) but not the microbial community. Thus, the knowledge about the behavior of the ruminal microbial community during the adaption phase is low, as only adaption to a dual-flow continuous culture

system was investigated (Ziemer et al., 2000). In this study, differences in the composition of the microbial community at the end of the adaption phase (after 240 h) and the inoculum were identified. But, in total, this *in vitro* system supported a functional community structure similar to that found in the rumen. Nevertheless, it is very important to know how the species react during adaption to the Rusitec system and which species are able to survive in this *in vitro* system.

Results from the current thesis (Exp. 1) and from Prevot et al. (1994), showed a decrease in total bacteria and protozoa numbers during the first two days of a Rusitec run. In Exp. 1 the decrease was irrespective of the incubated silage since similar results were observed in the blank fermenters that contained only the solid phase from the rumen without silage. The observed decrease on group levels of both studies (the current thesis and Prevot et al. 1994) was similar at species level. Prevot et al. (1994) reported that the microbial community observed in the Rusitec after the adaption phase was similar to that in defaunated animals. The results observed in the current thesis showed a different composition of microbial community at the end of the adaption phase, as compared to the inoculum. But Martínez et al. (2010a, 2010b) reported that the ruminal microbial community present in the Rusitec was similar to that in sheep, during the incubation of high forage sources-diets.

Thus, to evaluate the results which are generated with the Rusitec, it is important to know that the composition of ruminal microbial community is different to that found *in vivo*, however, under certain conditions the results can be transferred to the animal. In summary, the Rusitec is a good *in vitro* system to describe the effect of different feed stuffs or feed additives on the ruminal microbial community. This is also shown in Section 2.2, in which the effect of silage on the ruminal microbial community of all three experiments (two *in vitro* and one *in vivo* experiment) in the current thesis will be discussed.

### **2.1.2 ANALYSIS OF MICROBIAL POPULATIONS**

Prior real time qPCR, the microbial DNA was extracted from fermenter liquids as well as from feed residues (Exp. 1 and 2), and the liquid and solid fraction in the rumen (Exp. 3). Before samples of the experiments (Exp. 1-3) were extracted, different extraction protocols and methods were tested with rumen contents (Table 2). Furthermore, at the beginning of every laboratory phase, the extraction method was optimized with pool samples of the corresponding experiment. Thus, some extractions steps were adjusted to obtain DNA of sufficient yield and quality. This is very important, since the DNA yield and quality, as well as the absolute microbial numbers, depend on the DNA extraction method (Henderson et al., 2013). In order to obtain a representative overview of the ruminal microbial community, it is important to extract a high amount of DNA of good quality covering the whole diversity of microbial cell wall types commonly found in the rumen. In the current thesis, the adjustments of the extraction protocol were different between liquid and solid phases, since the physical properties of both phases were

different. For evaluation of the extraction method, the integrity as well as the purity of the extracted DNA was measured.

TABLE 2: Spectrophotometrically measurements (NanoDrop-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA) of DNA purity and concentration in DNA extracts obtained by different extraction protocols (gray shaded areas indicate the extraction protocols that were used in the current thesis).

Extraction protocol	Methodical variations (RBB+C <sup>1</sup> ):		Substrate	Nucleid acid concentration (ng/mg)	OD <sub>260/280</sub>	OD <sub>260/230</sub>
	Amounts of zirconia beads	Time and speed in the FastPrep Instrument <sup>2</sup>				
RBB+C	0.15 g of 0.1 mm and 0.05 g of 0.5 mm	40 sec. at level 4	liquid fraction <sup>3</sup>	112.7	1.85	2.05
RBB+C	0.05 g of 0.5 mm	40 sec. at level 4	solid fraction <sup>4</sup>	74.4	1.82	1.84
RBB+C	0.05 g of 0.5 mm	40 sec. at level 4	feed residues <sup>5</sup> of CS <sup>6</sup>	114.5	1.82	1.8
RBB+C	0.05 g of 0.5 mm	40 sec. at level 4	feed residues of GS <sup>7</sup>	90.71	1.86	1.93
RBB+C	0.3 g of 0.1 mm and 0.1 g of 0.5 mm	40 sec. at level 6	liquid fraction	45.2	1.96	0.76
RBB+C	0.3 g of 0.1 mm and 0.1 g of 0.5 mm	40 sec. at level 6	solid fraction	51.4	1.92	1.85
RBB+C	0.3 g of 0.1 mm and 0.1 g of 0.5 mm	10 sec. at level 4	liquid fraction	41.8	1.91	1.23
RBB+C	0.3 g of 0.1 mm and 0.1 g of 0.5 mm	10 sec. at level 4	solid fraction	52.9	2.01	1.3
QIAamp DNA Stool Mini Kit <sup>8</sup>	-	-	liquid fraction	11.3	6.57	2.33
QIAamp DNA Stool Mini Kit	-	-	solid fraction	39.5	2.33	2.04

<sup>1</sup>repeated bead beating plus column method (Yu and Morrison, 2004); <sup>2</sup>MP Biomedicals, Eschwege, Germany; <sup>3</sup>liquid fraction obtained from the rumen of a Friesian cow; <sup>4</sup>solid fraction obtained from the rumen of a Friesian cow; <sup>5</sup>feed residues after *in situ* incubation in a Friesian cow; <sup>6</sup>corn silage; <sup>7</sup>grass silage; <sup>8</sup>protocol for pathogen detection (Qiagen, Hilden, Germany).

In all three experiments (Exp. 1-3), the same extraction protocol (RBB+C method) from Yu and Morrison (2004) was used, with little adjustments for the individual experiments which are described in the corresponding manuscripts. Further studies (Henderson et al., 2013; Yu and Morrison, 2004) indicated that the RBB+C method used in the current thesis is suitable for this kind of analysis. Furthermore, Henderson et al. (2013) determined the effects of 15 different DNA extraction methods on DNA quality and quantity, as well as on absolute microbial numbers and relative microbial community composition, and reported that the RBB+C method produced the largest specific DNA yields with good integrity, and which were also well suitable for PCR. In order to compare Exp. 1-3 of the current thesis, the same extraction protocol must be used. Using different extraction methods could lead to different DNA yield and quality und thus could have an influence on the results of real time qPCR (Henderson et al., 2013).

For identification of the ruminal microbial community, sequence analysis of 16S rRNA, 18S rRNA (protozoa), and methyl coenzyme-M reductase (*mcrA*) genes, as well as the trimethylamine-methyltransferase (*mtt*) gene of Rumen Cluster C (RCC) was used. It is well known that small ribosomal subunits of prokaryotes (16S) and eukaryotes (18S) occur in all

organisms and have high variable regions allowing for identification (Ludwig and Schleifer, 1994; Woese, 1987) of single species or groups. The *mcrA* gene encodes the alpha subunit of the methyl-coenzyme M reductase, which catalyzes the last step in methanogenesis and, thus, is present in all methanogens (Friedrich, 2005). The *mtt* gene encodes the methyltransferase, which initiates methanogenesis from trimethylamine (Krätzer *et al.*, 2009) and, thus, is present in methylotropic methanogens that use methylamines as their major energy and carbon source, like the RCC.

It was assumed that the respective gene copy occurred once per cell. Thus, for each copy number detected in real time qPCR, one organism was counted. However, there is a possibility that a single cell carries several copies of the 16S or 18S rRNA gene, in dependence of the species and growth stage (Fogel *et al.*, 1999; Ogata *et al.*, 1997; Peterka and Avguštin, 2001; Sylvester *et al.*, 2009; Tajima *et al.*, 2001). Case *et al.* (2007) reported an average of 4.2 copies per genome after the evaluation of the 16S rRNA gene across 111 bacterial genomes. Thus, in the current thesis the abundance of some species could be overestimated. But, until now, in most studies that investigated the ruminal microbial community, the repeated occurrence of gene copies per cells were not considered since the numbers of gene copies per cell are known only for a few ruminal species (Acinas *et al.*, 2004; Fogel *et al.*, 1999). Even in the rRNA Operon Copy Number Database (Klappenbach *et al.*, 2001) only the copy numbers for *F. succinogenes* (3 copies per cell), *R. albus* (4 copies per cell), and *S. ruminantium* (7 copies per cell) are registered. Thus, the number of *S. ruminantium* seems to be overestimated by using this approach, and it explains the high numbers of *S. ruminantium* found in all three experiments (Exp. 1 - 3) as compared to the other species.

## **2.2 INFLUENCE OF SILAGE ON RUMINAL MICROBIAL COMMUNITY**

The first two experiments (Exp. 1 and 2) were carried out in the Rusitec system. The last experiment (Exp. 3) was conducted to evaluate the effect of different silages on the ruminal microbial community *in vivo*. In the following, the fermenter liquid from the *in vitro* experiments will be referred to as liquid fraction and the feed residues as solid fraction, similar to the fractions in Exp. 3, to simplify matters. In contrast to the *in vitro* experiments (Exp. 1 and 2), the *in vivo* experiment (Exp. 3) contained concentrate in the rations to meet the requirements of the lactating dairy cows. But the ratio and composition of concentrate in the rations was identical and the rations were isonitrogenous. Nonetheless, interactions between silage and concentrate could occur and, if necessary, will be discussed at the species level. For all experiments silage of good quality were used.

### TOTAL BACTERIA

In all three experiments, the total bacteria were minimally affected by silage, since total bacteria were affected only in Exp. 2 in the liquid fractions and in the solid fraction in Exp. 3. Most of the bacteria are associated with feed particles (Craig et al., 1987a; Olubobokun et al., 1988), and only in Exp. 3, effects were found in the solid fraction. If the total bacteria were affected, then higher numbers would be found with CS.

Similar to the abundance of total bacteria, in Exp. 1, the total gas production was not affected while in Exp. 2 a higher amount of total gas production was found during incubation of CS as compared to GS. Thus, higher amounts of total bacteria leads to higher total gas production. The higher amount of readily fermentable carbohydrates in CS, as compared to GS, could enhance the abundance of total bacteria. Similar results were reported by Lettat et al. (2013). The authors replaced alfalfa silage with CS in the rations of dairy cows and found higher numbers of total bacteria with higher amounts of CS in the ration. Benchaar et al. (2007) also found greater abundance of total bacteria in dairy cows fed with CS, as compared to those fed alfalfa silage based ration. Contrasting results were found by Staerfl et al. (2012) who recorded higher numbers of total bacteria in ruminal fluid of fattening bulls immediately after slaughter, that were fed with a GS-based ration as compared to bulls fed a CS-based ration. Unfortunately, the authors did not investigate the solid fraction and took samples at one time point only.

The reason for the marginal differences of ruminal bacteria abundance between the silages could be the diversity of the ruminal bacteria, which comprise a wide range of different species that have good adaption to different feeding conditions.

### PROTOZOA

The effect of silage on the protozoa population in the current thesis was clear. In two of three experiments, the protozoa were enhanced by CS. Only in Exp. 1 no effect of silage was detected, but numbers also tended to be higher with CS in both fractions. Contrasting results were found by Benchaar et al. (2007) and Staerfl et al. (2012). The latter found no effect of silage in fattening bulls right after slaughter, whereas Lettat et al. (2013) found higher numbers of protozoa in dairy cows fed with alfalfa silage as compared to CS.

The protozoal community in the rumen comprise a wide range of species with amylolytic, as well as proteolytic (Jouany, 1996) and cellulolytic activities (Williams and Coleman, 1997). In this thesis, it is possible that protozoa species with amylolytic activity may have been promoted by the corn starch. Due to the primers which were used in both experiments, it is not possible to describe single strains since detection was only performed on the group level.

Protozoa engulf and digest large numbers of bacteria as their source of protein (Coleman, 1986; Jouany, 1996). Thus, higher numbers of total bacteria in the solid fraction of Exp. 3 could have promoted the numbers of protozoa. This assumption is supported by the positive correlation

between numbers of protozoa and total bacteria in the solid fraction that was detected in Exp. 3. In Exp. 1 and 2, no correlation between the abundance of protozoa and total bacteria was found. But as discussed above the abundance of protozoa *in vitro* was substantially lower than *in vivo*.

#### METHANOGENIC ARCHAEA

The effect of silage on the methanogenic archaea was only found in Exp. 2. In both microbial fractions, higher numbers of methanogens were detected during incubation of GS. Accordingly, the percentage of methane in total gas was higher during incubation of GS. The total methane production per gram of fermented organic matter or per day was not different between the two silages (20 ml/g fermented organic matter for GS; 17 ml/g fermented organic matter for CS;  $P=0.383$ ). However, in Exp. 1, methane production was higher during the incubation of GS as well as the digestibility of organic matter, while the number of methanogens was not affected. Similar results were reported by Staerfl et al. (2012) who found no effect of silage on the numbers of methanogens in the ruminal fluid of fattening bulls, but did observe effects on methane production. Contrasting results were reported from Lettat et al. (2013) who found higher numbers of methanogenic archaea in lactating dairy cows fed the CS based ration as compared to rations with higher amounts of alfalfa silage, but lower methane production per kilogram of dry matter intake in CS based fed cows. It could be possible that the methanogen diversity and community structure is affected by the diet, but the density of rumen methanogens are consistent, as shown in a study from Hook et al. (2011). Furthermore, previous studies found no association between the abundance of methanogens and the methane production as described by Firkins and Yu (2015).

In principle, ruminal methanogenic archaea include a wide range of different species but most of them use hydrogen to reduce carbon dioxide to methane for generating energy. Thus, methanogenic archaea are not directly influenced by ration, but rather by fermentation end products from other ruminal microorganisms and the environment in the rumen.

Part of the methanogens is associated with protozoa since they produce hydrogen, whereby methanogens can increase their access to hydrogen which is an important substrate for them (Newbold et al., 2015; Tymensen et al., 2012). Thus, changes in the protozoa population could also lead to changes in the methanogenic community. Meta-analysis data from Newbold et al. (2015) showed a decrease in methane production in defaunated animals but no significant decrease in the numbers of methanogens. Results observed from Tymensen et al. (2012) showed that changes in the ration influenced the protozoal population and, similarly the overall methanogenic community. But it is not obvious if the change in the methanogenic community is due to protozoal disappearance and, thus, the loss of symbiotic protozoa species, or due to other changes in the rumen environment.

It is a fact, however, that ciliate endosymbiotic methanogens differ from rumen free-living methanogens species and that holotrich protozoa have different endosymbiotic methanogens than



entodiniomorphids (Newbold et al., 2015). Thus, it could be possible that changes in the protozoa population and ruminal environment lead to alteration in the methanogenic community, and thereby, change their potential to produce methane (Zhou et al., 2009).

In the current thesis, it was not possible to evaluate single species of the methanogenic archaea since detection was only performed on group level. Further research on species level is necessary to detect the interaction of protozoa and methanogenic archaea and the effect on methane production.

However, in the current thesis, numbers of protozoa were higher during incubation of CS whereas methanogens were higher during incubation of GS. It could be possible that the higher fiber content in GS and, thereby, the higher abundance of hydrogen-producing cellulolytic bacteria (e.g. *R. albus*), enhanced the abundance of methanogens.

#### RUMEN CLUSTER C

The RCC clade of *Thermoplasmata* is a novel group of methylotropic methanogens which use methylamines as their major energy and carbon source. Thus, the RCC occupy a niche separate from other rumen methanogens (Poulsen et al., 2013). Rumen bacteria can form trimethylamine from glycine betaine and choline (Mitchell et al., 1979; Neill et al., 1978) which can then be used by the RCC to produce methane. Thus, the rations fed to cows have only a small influence on the abundance of RCC. However, there is some evidence that the glycine betaine concentration in grass could be higher than in corn (Chendrimada et al., 2002; Lerma et al., 1991). This would be in accordance with results of the current thesis, since, in Exp. 2, higher numbers were found in both fractions during incubation of GS, while in Exp. 3, no effect of the silage was detected but numerically higher numbers were recorded for GS. Furthermore, it could be possible that the higher numbers of RCC in Exp. 2 during incubation of GS are responsible for the higher amount of methane, as compared to CS. RCC were only detected in Exp. 2 and 3.

#### F. SUCCINOGENES

This cellulolytic bacterium was expected to be at higher level with GS, since *F. succinogenes* is considered to be the predominant cellulolytic bacterium present in the rumen (Koike and Kobayashi, 2001). Furthermore, *F. succinogenes* is specialized for using only cellulose as an energy source since it does not contain the genes necessary to transport and metabolize the hydrolytic products of non-cellulose polysaccharides (Suen et al., 2011). The numbers of enzymes which are produced by this organism, which are capable of degrading a wide array of polysaccharides, are only for removing carbohydrates like xylan in order to gain access to cellulose. But in Exp. 1, a numerically higher abundance was detected in both fractions during incubation of CS. In Exp. 2, no effect of silage was detected in the liquid fraction, while in the solid fraction higher numbers of *F. succinogenes* were found during incubation of CS. In Exp. 3, higher numbers were found in liquid fraction of cows fed on the GS based ration, while in the

solid fraction, contrasting results were found. Contrary results are reported from Lettat et al. (2013) and Staerfl et al. (2012). The authors (Lettat et al., 2013) replaced alfalfa silages with CS in the rations of lactating dairy cows, and found no effect of the silage on the abundance of *F. succinogenes* in ruminal fluid. Staerfl et al. (2012) fed fattening bulls with either CS or GS, and took samples from ruminal fluid right after slaughter. They also found no effect of silage on the abundance of *F. succinogenes*. One explanation for these findings could be that Staerfl et al. (2012) only investigated the ruminal fluid, but *F. succinogenes* does not excrete significant amounts of cellulases into its environment. Furthermore, degradation of cellulose requires attachment of *F. succinogenes* to the cellulose surface (Suen et al., 2011). Thus, the effect of silage on *F. succinogenes* in the rumen fluid is restricted.

One explanation for the higher numbers of *F. succinogenes* in CS, as compared to GS, in the current thesis could be the differences of cell wall structure between C<sub>3</sub> and C<sub>4</sub> plants. C<sub>4</sub> plants like corn possess a much tougher cell wall as compared to C<sub>3</sub> plants from which GS is obtained (Akin, 1989). Suen et al. (2011) showed that the cellulose degradation strategy by *F. succinogenes* is not obvious and stands in strong contrast to strategies used by other cellulolytic microorganisms. Furthermore, during *in situ* incubation of orchard grass hay, Shinkai and Kobayashi (2007) reported a higher abundance of *F. succinogenes* in less easily degradable stems than *R. flavefaciens*. Kozakai et al. (2007) found higher numbers of *F. succinogenes* on corn steams, as compared with *R. albus* and *R. flavefaciens*. In summary, our results give strong indication that *F. succinogenes* has an advantage in degrading tougher cell walls such as those found in corn.

#### *R. ALBUS* AND *R. FLAVEFACIENS*

As expected, both cellulolytic species were enhanced by GS. However, in Exp. 1 and 2, *R. albus* was only affected by silage in the liquid fraction. The lack of effect of silage on the abundance of *R. albus* in the solid fraction of both experiments could be a result of sampling time, which is already discussed in section 2.1.1. In brief, it is possible that the *R. albus* are already detached since the highest numbers for particle associated microorganisms in the rumen of dairy cows were found one hour after feeding, and were at their lowest after 10 h (Craig et al., 1987a). Indeed, in Exp. 3, no effect of sampling time was observed on the abundance of *R. albus* in the solid fraction, but in this experiment the cows were fed *ad libitum*. Thus, fresh substrate was available at any time, which is shown by the dry matter intake. *In vitro* fresh substrate was only available after 24 h.

In Exp. 3, both species (*R. albus* and *R. flavefaciens*) were higher in both fractions in cows fed on the GS-based ration. In the *in vitro* studies (Exp. 1 and 2), *R. flavefaciens* could not be quantified since the primers used for the *in vivo* trial resulted only in unspecific PCR products, irrespective of the conditions chosen for amplification. Thus, *R. flavefaciens* were only detected *in vivo*.

### *S. RUMINANTIUM*

*S. ruminantium* showed no clear preference for silage in this thesis. In Exp. 1, no effect of silage was detected, while in Exp. 2, the numbers of *S. ruminantium* in the solid fraction were higher during incubation of CS. In Exp. 3, higher numbers were found in the liquid fraction of cows fed on the GS-based ration. Furthermore, the effect of silage was very small. This might be explained by the ability of this species to utilize a wide range of degradation products as substrates. Cotta (1988) showed that *S. ruminantium* is able to grow in starch-containing medium but produces little amylase, and that the growth is much less than observed when glucose is provided as the energy source. The authors speculated that *S. ruminantium* incompletely hydrolyzes starch. Furthermore, the same author reported in a further study (Cotta, 1992) that co-cultivation of *S. ruminantium* with either *Prevotella ruminicola* or *Butyrivibrio fibrisolvens* enhanced the growth of *S. ruminantium* and decreased the accumulation of maltooligosaccharides, which occur at a higher level in the culture of *B. fibrisolvens* alone. Russell (1985) showed that *S. ruminantium* used cellulodextrins, a degradation product arising during fermentation on cellulose by cellulolytic species. This could be the reason for higher numbers in the liquid fraction of cows fed the GS-based ration in Exp. 3, since the higher amount of degradable fiber in GS, as compared to CS, may have made more degradation products of fiber available for *S. ruminantium*. To conclude, the abundance of *S. ruminantium* depends on the abundance of other ruminal bacteria and their fermentation products, which are then used by *S. ruminantium* as the energy source. Thus, the different compositions of the ruminal community caused by different silage and rations, as well as the different systems (*in vivo* and *in vitro*) of the current thesis, could be the reason behind the different response of *S. ruminantium* during the experiments.

### *R. AMYLOPHILUS*

The effect of silage on *R. amylophilus* was only observable in Exp. 3. In Exp. 1, *R. amylophilus* was detected during the first 48 h of incubation, after which, generation of non-specific PCR products did not allow quantifications of this species. Similar problems occurred in Exp. 2. One possibility could be a substantial decrease in numbers of *R. amylophilus* due to what may indicate a lack of adaption to the Rusitec. But, in another Rusitec study (Narvaez et al., 2013), the authors could quantify *R. amylophilus* after 16 days of incubation. One explanation could be that in this study (Narvaez et al., 2013) other primer pairs were used. This indicates that not just the adaption of *R. amylophilus* to the Rusitec system, but also the primer pairs which were used in the current thesis, could be reasons behind the decrease in numbers. Other authors (Patra and Yu, 2013) also used the primer pairs from Tajima et al. (2001) which were used in the current thesis. However, this study (Patra and Yu, 2013) was carried out in 120-mL bottles which were incubated in a warm water bath for only 24 h.

In Exp. 1, *R. amylophilus* was enhanced during the first 48 h by CS. In contrast, in Exp. 3, higher numbers were found with GS. *R. amylophilus* requires starch or maltose as an energy source and not glucose (Anderson, 1995; Cotta, 1988).

However, during the incubation of silage alone, as in Exp. 1, the starch from CS obviously enhanced the growth of this bacterium. But *in vivo* the combination of silage and concentrate leads to a different reaction. Furthermore, in Exp. 3, only two of the three cows showed a higher abundance of *R. amylophilus*, during the incubation of GS. Thus, the effect of silage, *in vivo*, has another influence on the abundance of *R. amylophilus*, just as *in vitro*. But the current thesis did not determine whether the animals or concentrate are responsible for this differences.

#### *P. BRYANTII*

The effect of silage on the abundance of *P. bryantii* was clear in this thesis. In all experiments, higher numbers of this species were found in both fractions with GS. This was not astonishing since *P. bryantii* ferments peptides and amino acids (Ling and Armstead, 1995) and is also involved in the degradation of hemicelluloses (Matsui et al., 2000; Miyazaki et al., 1997). The higher CP and fiber content in GS, as compared to CS, enhanced the numbers of *P. bryantii*. Similar results were found by Witzig et al. (2010a) who also found a higher proportion of this species during *in vitro* incubation of GS, as compared to CS.

The results of Exp. 2 showed that supplementation of CS with peptone or amino acids increased the number of *P. bryantii*, but not to the level seen for GS. Thus, the higher CP content (higher content of true protein and, thus, peptides and amino acids) of GS alone could not be the reason for the higher abundance of *P. bryantii*, rather, the higher content of degradable fiber in GS also played a role.

#### *C. AMINOPHILUM*

*C. aminophilum* belongs to the class of hyper ammonia-producing bacteria (HAB) which use peptides or amino acids as their sole energy source and produce up to 20-fold more ammonia than other ammonia-producing ruminal bacteria (Paster et al., 1993). Thus, it was expected that *C. aminophilum* would display higher abundance with GS as compared to CS, and that a higher ammonia concentration would occur in the presence of this bacterium. However, higher numbers of *C. aminophilum* after incubation of GS were observed only in the solid fraction in Exp. 1, while in Exp. 3, no differences were detected between silages. One possibility could be that the HAB are much less numerous than others (Russell et al., 1988) and thus the concentration of *C. aminophilum* in the rumen may have been too low for detection. Results from Exp. 1 showed a significant increase in the numbers of *C. aminophilum* during the adaption phase *in vitro*, which indicates that this species has an advantage over others when it comes to adapting to the Rusitec. Besides, the different rations used can also help explain the different findings between Exp. 1 and 3.

In contrast to Exp. 1, in Exp. 2, higher numbers of *C. aminophilum* were observed in the liquid fraction during incubation of CS, and a higher ammonia concentration was seen in fermenter liquids during incubation of GS. Thus, results of the current thesis showed that *C. aminophilum* was not clearly enhanced by GS or CS. Furthermore, higher ammonia concentration could not be produced from this species which is possibly a result of other ammonia producing bacteria like *Peptostreptococcus anaerobius* or *Clostridium sticklandii* that also count among HAB but were not analyzed in the current thesis.

### **2.3 FERMENTATION CHARACTERISTICS AND EFFICIENCY OF MICROBIAL CRUDE PROTEIN SYNTHESIS IN THE RUSITEC EXPERIMENTS**

Basically, during the fermentation of fiber, the major volatile fatty acid produced is acetate, while during the fermentation of starch and highly soluble carbohydrates, the production of propionate and butyrate increases, thus decreasing the acetate to propionate ratio. However, in Exp. 1 a higher propionate production was found during incubation of GS, and no differences were observed between the two silages in Exp. 2. Furthermore, in Exp. 1, acetate production was not affected by silage, while in Exp. 2, more acetate was found for CS. Thus, in both experiments, the acetate to propionate ratio was higher during incubation of CS. The butyrate concentration was higher during incubation of CS in both experiments (Exp. 1 and 2). Similar to the results from Exp. 1, Hildebrand et al. (2011b) also found no effect of silage on acetate production in the Rusitec, but the authors also reported a higher propionate production during incubation of CS as compared to GS. Thus, contrary to our results, Hildebrand et al. found in both studies (2011b, 2011c) a higher acetate to propionate ratio during incubation of GS, as compared to CS. Similar to results from Boguhn et al. (2012) and Hildebrand et al. (2011b, 2011c), the degradation of organic matter was higher during incubation of GS in both experiments (Exp. 1 and 2). But the abundance of total bacteria was less affected by silage and higher during incubation of CS. One explanation for these findings could be that not only the total bacteria but methanogens, protozoa, and fungi are also involved in the degradation of organic matter. Calculations on the overall numbers of microorganisms in fermenters (sum of total bacteria, methanogens, and protozoa) showed higher numbers of microorganisms in the liquid fraction only during the first four hours after changing the feed bag for GS, in Exp. 1. Afterwards, higher numbers were found for CS. On an average of all sampling times, the numbers in both silages were similar (Exp. 1). In Exp. 2, the sum of total bacteria, methanogens, and protozoa was higher during incubation of CS as compared to GS. Indeed, the abundance of fungi was not detected in the current thesis since unspecific products were generated during real time qPCR. But, compared to bacteria, fungi are much less abundant in the ruminal community. Thus, the missing data on the abundance of fungi should not affect the results calculated in the current thesis. However, it would be an interesting topic for further

research since fungi are very important for fiber degradation, and it could be possible that fungi have great influence in relation to their abundance in the rumen.

However, the numbers of total bacteria and the sum of overall microorganisms are not consistent with the efficiency of microbial CP synthesis in the current thesis. In both *in vitro* experiments (Exp. 1 and 2), the efficiency of microbial CP synthesis was higher during incubation of GS as compared to CS, while the number of total bacteria was less affected by silage, or higher during incubation of CS. Similar results were observed from Martínez et al. (2010a, 2010b), who found a significant effect of forage source on the efficiency of microbial CP synthesis in the Rusitec, but no influence on the numbers of total bacteria. There are several reasons behind the different findings of total bacteria numbers and efficiency of microbial CP synthesis. First of all, the samples used to calculate the efficiency of microbial CP synthesis were collected from the fermenter liquid once daily in the morning from Day 7 - 13 of each Rusitec run, and only on day 13 (at the end of each Rusitec run) from the feed residues which were incubated at this time for 24 h and 48 h, respectively. Thus, samples of fermenter liquids were taken at a time when the concentration of total bacteria was minimal as shown in Exp. 1. Furthermore, samples from the solid fraction were taken on only one day, and after 24 h and 48 h, which may be too late to investigate all solid associated microorganisms as previously discussed (Chapter 2.1.1). Additionally, the CP content of microorganisms could be different among ruminal microbial groups as well as the copy numbers of the rRNA gene per cell. All in all, there are several factors that can be responsible for the differences between the numbers of total bacteria, as well as the sum of microbial fraction and the efficiency of microbial CP synthesis, and this needs further research.

The higher efficiency of microbial CP synthesis for GS than for CS is in accordance with former *in vitro* studies (Boguhn et al., 2012; Hildebrand et al., 2011c), but in contrast to results which were observed *in vivo* (Givens and Rulquin, 2004). In previous *in vitro* studies (Hildebrand et al., 2011a, Hildebrand et al., 2011c), it was supposed that the absence of the rumino-hepatic circulation in *in vitro* studies resulted in lower nitrogen availability for the microorganisms, that this could be the reason behind the lower efficiency of microbial CP synthesis *in vitro* as compared to *in vivo*. The supplementation of urea to CS in the Rusitec increased the efficiency of microbial CP synthesis, but the high value detected for GS was not achieved (Hildebrand et al., 2011c). This could be the result of inadequate nitrogen sources, since the preference of species for different nitrogen forms has long been observed (Argyle and Baldwin, 1989; Carro and Miller, 1999; Griswold et al., 1996). Thus, in the current thesis (Exp. 2), the effect of different nitrogen forms added to the CS has been investigated. However, the results showed that neither the amount of available nitrogen nor the source of nitrogen are the only limiting factors for lower efficiency of microbial CP synthesis obtained within CS and compared to GS *in vitro*. A further possibility

could be the synchronization of the degradation rate of carbohydrates and proteins and, therefore, the supply of energy and nitrogen for the ruminal microbial community (Soto et al., 1994) in the Rusitec during incubation of CS or GS. The synchronized supply of nitrogen and energy could increase the efficiency of microbial CP synthesis. During the fermentation of GS, the nitrogen will be released slowly and not as fast as during the fermentation of urea or other nitrogen forms which were added on the top, as in Exp. 2. This slow release of fiber-bound nitrogen during fiber degradation in GS is probably more synchronized with the supply of energy, as in the case of adding nitrogen on the top of CS.

Another reason behind the inconsistent results of the efficiency of microbial CP synthesis could be the abundance of protozoa. In both experiments (Exp. 1 and 2), incubation of CS enhanced the protozoal population. Due to the fact that protozoa engulf and digest bacteria (Jouany, 1996) they have an effect on the efficiency of microbial CP synthesis. As shown by Jouany (1996) and Newbold et al. (2015), the efficiency of microbial CP synthesis was higher in defaunated animals. This is in line with results from the current thesis, since in both experiments, the protozoa numbers were negatively correlated with the efficiency of microbial CP synthesis (Exp. 1:  $r=-0.75$  in the solid fraction;  $r=-0.88$  in the liquid fraction). Furthermore, protozoa have a higher energy requirement than bacteria for maintenance due to longer generation intervals and motility (Hackmann and Firkins, 2015; Williams and Coleman, 1997). Thus, the abundance of protozoa is supposed to have a negative effect on the energetic efficiency in the rumen. The higher numbers of protozoa in CS could, therefore, also decrease the efficiency of microbial CP synthesis in the current thesis.

Karsli and Russell (2002) reported that for maximal microbial protein synthesis, apart from non-protein nitrogen, amino acids, peptides, as well as a mixture of structural and non-structural carbohydrates are necessary. But the results of Exp. 2 showed that this is not entirely correct. Indeed, the supplementation of amino acids or peptides to CS, which corresponds to the requirements from Karsli and Russell (2002) enhanced the efficiency of microbial CP synthesis but not to a maximum since the efficiency of microbial CP synthesis in GS was still higher.

In summary, there are several possibilities until now that could be the reason behind the differences in efficiency of microbial CP synthesis *in vitro* and *in vivo*. Thus, further research is necessary to clarify this issue.

The sum of microbial protein from fermenter liquids and feed residues (microbial protein synthesis) was higher in both experiments (Exp. 1 and 2) during incubation of GS as compared to CS (Exp. 1: 1379 mg per day for GS and 849 mg per day for CS;  $P<0.0001$ ). However, the amount of total bacteria, as well as the sum of total bacteria and methanogens, was similar in the silages, or somewhat higher during incubation of CS. As mentioned above, the different CP

content of the microorganisms as well as the numbers of rRNA genes per cell could be the reason for these findings.

On assessing the different nitrogen sources added to CS (Exp. 2), it is clear that the supplementation of urea and peptone numerically increased the microbial protein synthesis, as compared to GS. Similarly, the degradation of CP and organic matter was higher in both CS variants as well as in GS. This indicates that the additional nitrogen availability of the supplemented CS enhanced the microbial activity. Thus, incubation of CS alone leads to lack of nitrogen. But, results of the current thesis showed that it is not only the amount or the form of nitrogen which are responsible for the inconsistent results *in vitro* and *in vivo*. Furthermore, as shown in Exp. 1, the composition of the ruminal microbial community changes during the adaption period. Thus, a different community occurs *in vitro* as compared to *in vivo*. This could also have an influence on the efficiency of microbial CP synthesis, since Boguhn et al. (2012) and Witzig et al. (2015) showed that difference in feeding among donor animals result in different ruminal microbial communities with varying efficiencies of microbial CP synthesis.

## **2.4 DIURNAL CHANGES**

In the current thesis, significant diurnal changes of the ruminal microbial community were observed *in vitro* (Exp. 1) and *in vivo* (Exp. 3). Diurnal changes were not of interest in Exp. 2, but due to the results of Exp. 1 which indicated significant changes over time, samples were collected at different time points to get a representative result of the influence of different nitrogen sources on the rumen microbial community. Prior DNA extraction samples taken at different time points were pooled.

Results of the current thesis are in accordance with other studies, which have also indicated diurnal changes of the ruminal microbial community *in vitro* (Belanche et al., 2013) and *in vivo* (Bryant and Robinson, 1968; Clarke, 1965; Craig et al., 1987a; Craig et al., 1987b; Dehority and Tirabasso, 1989; Dehority and Mattos, 1978; Koike et al., 2003; Koike et al., 2014; Potter and Dehority, 1973; Warner 1966; Welkie et al., 2010).

In the liquid fraction of Exp. 1, almost all species which were investigated showed highest numbers two hours after providing a new feed bag, and showed the same level after 24 h, as the reading before fresh substrate was added to the fermenter. Only protozoa and methanogens showed contrasting behavior. The decreasing numbers of protozoa in liquid fraction could be a result of migration from the liquid fraction to newly introduced feed particles (Santra et al., 1998; Valdez et al., 1977). Similar growth curves were observed from Belanche et al. (2013) who found the highest numbers of bacteria during the first few hours after changing the feed bag, and decreasing numbers of protozoa and methanogens in fermenter liquids. This growth curve indicated that adding fresh substrate *in vitro* enhanced the growth of bacteria, rapidly.



*In vivo* (Exp. 3) diurnal changes of microbial species did not show the same consistent increase of almost all species, after the highest dry matter intake, like *in vitro*. The dry matter intake was highest between 8.30 am and 12.30 pm and, during this period, a decrease in the species was observed. This postprandial decrease could be a result of dilution of rumen content via feed, water, and saliva (Bryant and Robinson, 1968; Warner, 1966) since the decrease in pH and increase in concentration of volatile fatty acids in the rumen indicated a higher microbial activity during this time period. But, otherwise, the species and groups investigated *in vivo* (Exp. 3) did not show the uniform growth curves as observed *in vitro* (Exp. 1). Thus, the composition changes in the ruminal microbial community during the day are influenced by feed and water intake. Therefore, the relative abundance of a single species to the total rumen bacteria also differed, as shown in Fig. 1 for Exp. 1 and Fig. 2 for Exp. 3. Similar to the results of absolute quantification, lower effects were observed on the solid fraction. This is in accordance with results observed from Welkie et al. (2010) who found lesser changes in composition of solid-associated microorganisms within and across feeding cycles, as compared to the changes of liquid-associated microorganisms using ARISA. Nevertheless, differences in the relative abundance were observed *in vivo* and *in vitro*, thus underlining the need for repeated sampling over time.

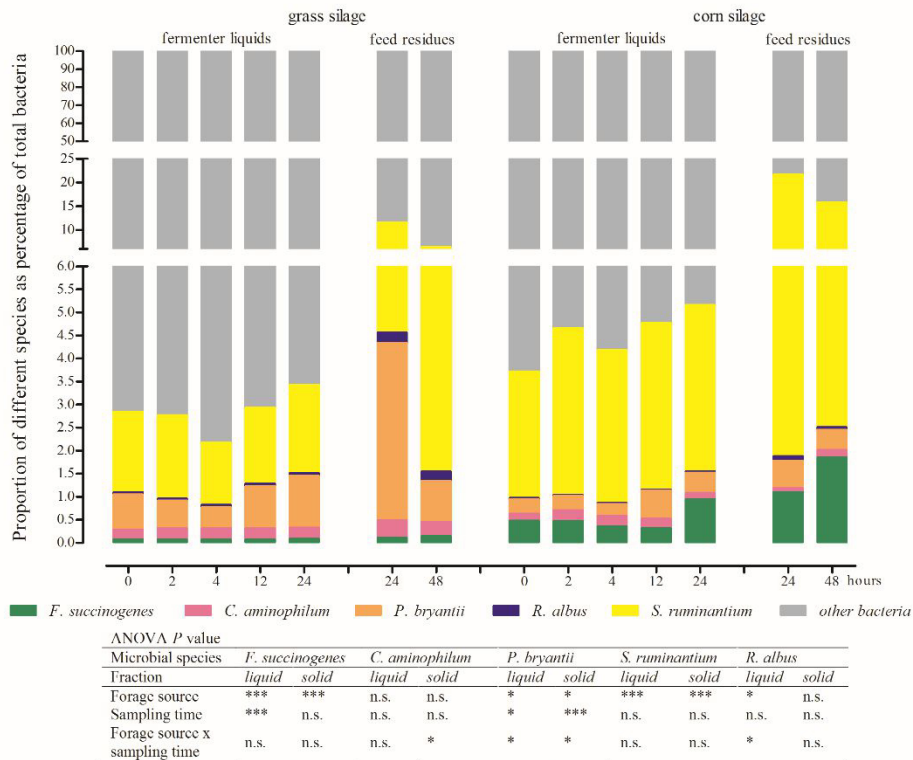


FIGURE 1: Proportion of *Fibrobacter succinogenes*, *Clostridium aminophilum*, *Prevotella bryantii*, *Ruminococcus albus*, and *Selenomonas ruminantium* in fermenter liquids and feed residues at different times of incubation in the second period of **Experiment 1** (as percentage of total bacterial 16S rRNA gene copy numbers; mean;  $n \geq 2$ ; \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ )

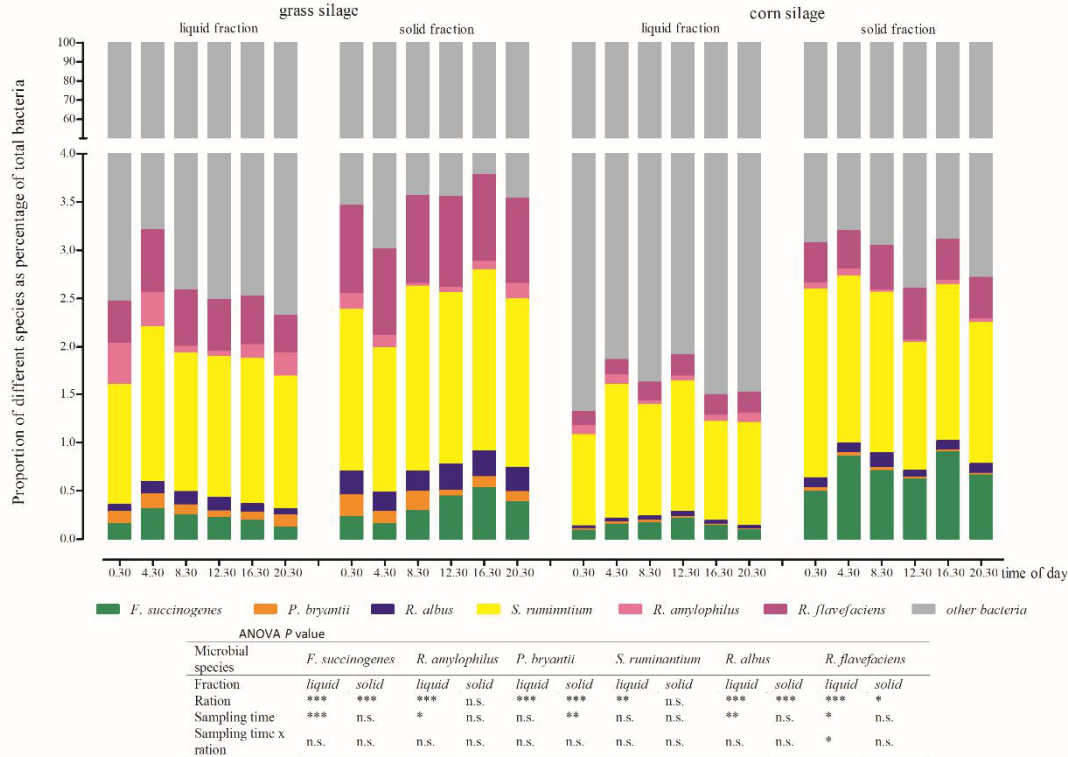


FIGURE 2: Proportion of *Fibrobacter succinogenes*, *Prevotella bryantii*, *Ruminococcus albus*, *Selenomonas ruminantium*, *Ruminobacter aminophilus*, and *Ruminococcus flavefaciens* in liquid and solid fraction at different sampling times in **Experiment 3** (as percentage of total bacterial 16S rRNA gene copy numbers; mean;  $n \geq 2$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ )

The results of the current thesis (Exp. 1 and 3) as well as from previous *in vivo* studies (Bryant and Robinson, 1968; Clarke, 1965; Craig et al., 1987a; Craig et al., 1987b; Warner, 1966) demonstrate that sampling at one time point showed only the diversity and composition of rumen bacteria at the moment of sampling, which may completely change within a few hours. For evaluating effects of different feed stuffs or feed additives on the ruminal microbial community, one sampling time per day is not sufficient. Furthermore, it is a serious mistake to believe, that one sampling time per day could adequately describe the effect on the ruminal microbial community. It is necessary to collect samples at different times over the day in order to obtain a meaningful result. As in the current thesis, a sampling interval of two or four hours would be adequate to get an overview of the ruminal microbial community.

The different effects of sampling time on the abundance of the ruminal microbial community in solid and liquid fractions underline the importance of sampling both fractions in order to obtain a complete compendium of the ruminal microbial community. Sampling of only one fraction could lead to an overestimation or underestimation of the tested aspect. Furthermore, microorganisms of the solid and liquid fraction are together responsible for fermentation.

In most cases, the silage had no effect on the diurnal changes since an interaction between sampling time and silage was only detected for the protozoa in both fractions, RCC in the solid

fraction, and *R. flavefaciens* in the liquid fraction *in vivo* (Exp. 3). *In vitro* (Exp. 1), the same was detected for *R. amylophilus* in the liquid fraction. Similar results were observed in a study by Belanche et al. (2013), which investigated the effect of red clover and ryegrass on the ruminal microbial community in the Rusitec, and found significant differences between forage sources only for a few sampling times within a sampling point. These results indicated that it is not just the silage or the ration which has an effect on the diurnal changes in ruminal microbial community, but rather the feed intake, feed passage rate, individual generation intervals of the species, and colonization mechanisms, as well as the association of microorganisms to feed particles, rumen wall, or liquid fraction.

## 2.5 CONCLUSIONS AND PERSPECTIVES

The ruminal microbial community *in vitro* as well as *in vivo* is able to deal with different silages but it shows diverse microbial structures with different fermentation end products depending on the silage. The effect of silage on total bacteria and methanogens was rather small since, for both groups, significant influences of the silage were found only in one experiment. But, for RCC, a novel group of methylotrophic methanogens, a promoting effect of the GS could be observed. Despite the protozoa comprising different species with proteolytic, amylolytic, and cellulolytic activity, this group appears to be enhanced by the CS. During incubation in the Rusitec, which leads to a significant increase of this group, the positive effect of CS was also detectable. As expected, the cellulolytic species *R. albus* and *R. flavefaciens* were both enhanced by the GS, as was *P. bryantii* which uses peptides and amino acids as energy source. The surprising fact was that in all experiments *F. succinogenes* was enhanced by CS and not, as expected, by GS. *R. amylophilus*, which was detectable only during the first 48 h *in vitro*, seems to be enhanced by the CS, while *in vivo* the combination of concentrate and GS favors this species. The effect of silage on the generalist *S. ruminantium* was different in all three experiments, as was the abundance of *C. aminophilum*, although the latter is a HAB and thus was expected to be enhanced by the GS. The results of the current thesis showed that different silages affected individual species differently, which led to the diverse composition of the ruminal microbial community with different fermentation end products. These differences could also influence the performance of the cow. Furthermore, the behaviors of species in the rumen were so far not well-known, although the rumen has been investigated for decades. Thus, further research is necessary to understand the functions of single species in the rumen within the ruminal microbial community.

For further information about the protozoal population and methanogens, it may be helpful to investigate the effects of silage at a species level. Both groups comprise a large number of species with obviously different substrate requirements and fermentation end products. Furthermore, it is already described that some methanogens are associated with protozoa, which also have an effect

on the fermentation of substrate in the rumen. For a better understanding of the interaction between these two groups and the function of protozoa, especially in fiber degradation, investigation at the species level is important.

In the current thesis, the fungi could not be investigated due to unspecific products during the real time qPCR. But, for a better understanding of the ruminal microbial community and fiber degradation, it is also important to investigate the ruminal fungi population.

During the experiments of the current thesis it became obvious that samplings at different time points over the day are inevitable in order to obtain meaningful results. Sampling at one time point can never completely describe the effect of silage on the ruminal microbial community, regardless of *in vitro* or *in vivo*. Spot-sampling shows only the momentary diversity of the ruminal microbial community, which can change a few hours later. To consider diurnal changes, it is not necessary to analyze every sampling time separately. Samples of different time points can be pooled prior to analysis to minimize the high costs of analyses.

The investigation of only the liquid fraction in the Rusitec or *in vivo* is not sufficient. Most of the microorganisms associated with feed particles are not considered if samples are collected only from the liquid fraction. Furthermore, the effect of silage on the ruminal microbial community is different between liquid and solid fractions. Additionally, diurnal changes occur between both fractions. Thus, observation of only one fraction cannot provide a complete overview of the ruminal microbial community.

The investigation of single species in pure culture can show the kind of substrates preferred by this species as well as the kind of fermentation end product produced. The same results can be different in mixed rumen cultures. Ruminal microbial species interact with each other with a complexity that cannot be simulated in pure cultures. Furthermore, in some cases, the microbial species are in competition for substrate, which also has an effect of the use of substrate and growth rate. Thus, studies which investigate the behavior of single species in mixed rumen culture are important for a better understanding of the complex rumen system. Therefore, other analyzing techniques as used in the current thesis could be helpful.

The composition of ruminal microbial community in the Rusitec was different from that in the inoculum. But, the tendency by which silage enhanced the abundance of species *in vitro* and *in vivo* was similar. Nevertheless, for some species, different reactions were observed during the three experiments. Therefore, during evaluation of results from the Rusitec it should be known that the composition of the ruminal community is different, and the use of this *in vitro* system should be well chosen in dependence on the experimental question.

Regarding the efficiency of microbial CP synthesis, there are still open questions as to why contrary results are observed *in vivo* and *in vitro*. Here, further research is necessary to generate similar results *in vivo* and *in vitro*, in order to obtain an *in vitro* system which describes the fermentation characteristics and the behavior of ruminal microbial community as closely as possible to the situation in the rumen. Results of the current thesis and previous studies showed that incubation of CS alone always leads to a nitrogen deficit for the microorganisms in the Rusitec which have a negative effect on the efficiency of microbial CP synthesis. The lack of rumino-hepatic circulation *in vitro* leads to a non-synchronized supply of energy and nitrogen. Thus, it is possible that the incubation of CS alone is not adequate for this kind of investigation. Moreover, further research is necessary to evaluate the use of the Rusitec system in investigating the efficiency of microbial CP synthesis, as well as to suggest other suitable *in vitro* systems is required.



## REFERENCES

- Abe, M., Kumeno, F. (1973). In vitro simulation of rumen fermentation: apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. *J. Anim. Sci.* 36, 941-948.
- Abrahamse, P. A., Vlaeminck, B., Tamminga, S., Dijkstra, J. (2008). The effect of silage and concentrate type on intake behavior, rumen function, and milk production in dairy cows in early and late lactation. *J. Dairy Sci.* 91, 4778-4792.
- Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V., Polz, M. F. (2004). Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J. Bacteriol.* 186, 2629-2635.
- Akin, D. E. (1989). Histological and physical factors affecting digestibility of forages. *Agronomy Journal* 81, 17-25.
- Amann, R. I., Ludwig, W., Schleifer, K. -H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143-169.
- Anderson, K. L. (1995). Biochemical analysis of starch degradation by *Ruminobacter amylophilus* 70. *Appl. Environ. Microbiol.* 61, 1488-1491.
- Argyle, J. L., Baldwin, R. L. (1989). Effects of amino acids and peptides on rumen microbial growth yields. *J. Dairy Sci.* 72, 2017-2027.
- Atasoglu, C., Valdés, C., Walker, N. D., Newbold, C. J., Wallace, R. J. (1998). De novo synthesis of amino acids by the ruminal bacteria *Prevotella bryantii* B14, *Selenomonas ruminantium* HD4, and *Streptococcus bovis* ES1. *Appl. Environ. Microbiol.* 64, 2836-2843.
- Bach, A., Calsamiglia, S., Stern, M. D. (2005). Nitrogen metabolism in the rumen. *J. Dairy Sci.* 88, E9-E21.
- Belanche, A., Lee, M. R. F., Moorby, J. M., Newbold, C. J. (2013). Comparison of ryegrass and red clover on the fermentation pattern, microbial community and efficiency of diet utilisation in the rumen simulation technique (Rusitec). *Animal Production Science* 53, 1052-1064.
- Benchaaar, C., Petit, H. V., Berthiaume, R., Ouellet, D. R., Chiquette, J., Chouinard, P. Y. (2007). Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *J. Dairy Sci.* 90, 886-897.
- Boguhn, J., Kluth, H., Rodehutsord, M. (2006). Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. *Journal of Dairy Science* 89, 1580-1591.
- Boguhn, J., Kluth, H., Bulang, M., Engelhard, T., Rodehutsord, M. (2010). Effects of pressed beet pulp silage inclusion in maize-based rations on performance of high-yielding dairy cows and parameters of rumen fermentation. *Animal* 4, 30-39.

- Boguhn, J., Kluth, H., Rodehutschord, M. (2006). Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. *J. Dairy Sci.* 89, 1592-1603.
- Boguhn, J., Strobel, E., Witzig, M., Tebbe, C. C., Rodehutschord, M. (2008). Description of the structural diversity of rumen microbial communities in vitro using single-strand conformation polymorphism profiles. *Arch. Anim. Nutr.* 62, 454-467.
- Boguhn, J., Zuber, T., Rodehutschord, M. (2012). Effect of donor animals and their diet on in vitro nutrient degradation and microbial protein synthesis using grass and corn silages. *J. Anim. Physiol. Anim. Nutr.*
- Brask, M., Lund, P., Hellwing, A. L. F., Poulsen, M., Weisbjerg, M. R. (2013). Enteric methane production, digestibility and rumen fermentation in dairy cows fed different forages with and without rapeseed fat supplementation. *Anim. Feed Sci. Technol.* 184, 67-79.
- Bryant, M. P., Robinson, I. M. (1968). Effects of diet, time after feeding, and position sampled on numbers of viable bacteria in the bovine rumen. *J. Dairy Sci.* 51, 1950-1955.
- Carro, M. D., Lebzien, P., Rohr, K. (1995). Effects of pore size of nylon bags and dilution rate on fermentation parameters in a semi-continuous artificial rumen. *Small Ruminant Res.* 15, 113-119.
- Carro, M. D., Miller, E. L. (1999). Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). *Br. J. Nutr.* 82, 149-157.
- Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., Kjelleberg, S. (2007). Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl. Environ. Microbiol.* 73, 278-288.
- Castillo-González, A. R., Burrola-Barraza, M. E., Domínguez-Viveros, J., Chávez-Martínez, A. (2014). Rumen microorganisms and fermentation. *Archivos De Medicina Veterinaria* 46, 349-361.
- Chendrimada, T. P., Neto, M. G., Pesti, G. M., Davis, A. J., Bakalli, R. I. (2002). Determination of the betaine content of feed ingredients using high-performance liquid chromatography. *J. Sci. Food Agric.* 82, 1556-1563.
- Chikunya, S., Newbold, C. J., Rode, L., Chen, X. B., Wallace, R. J. (1996). Influence of dietary rumen-degradable protein on bacterial growth in the rumen of sheep receiving different energy sources. *Anim. Feed Sci. Technol.* 63, 333-340.
- Cieslak, A., Zmora, P., Pers-Kamczyc, E., Stochmal, A., Sadowinska, A., Salem, A. Z. M., Kowalczyk, D., Zbonik, P., Szumacher-Strabel, M. (2014). Effects of two sources of tannins (*Quercus* L. and *Vaccinium vitis idaea* L.) on rumen microbial fermentation: An in vitro study. *Ital. J. Anim. Sci.* 13, 290-294.
- Clarke, R. T. J. (1965). Diurnal variation in the numbers of rumen ciliate protozoa in cattle. *N. Z. J. Agric. Res.* 8, 1-9.
- Coleman, G. S. (1986). The metabolism of rumen ciliate protozoa. *FEMS Microbiol. Lett.* 39, 321-344.



- Cotta, M. A. (1992). Interaction of ruminal bacteria in the production and utilization of maltooligosaccharides from starch. *Applied and Environmental Microbiology* 58, 48-54.
- Cotta, M. A. (1988). Amylolytic activity of selected species of ruminal bacteria. *Applied and Environmental Microbiology* 54, 772-776.
- Craig, W. M., Broderick, G. A., Ricker, D. B. (1987a). Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J. Nutr.* 117, 56-62.
- Craig, W. M., Brown, D. R., Broderick, G. A., Ricker, D. B. (1987b). Post-prandial compositional changes of fluid- and particle-associated ruminal microorganisms. *J. Anim. Sci.* 65, 1042-1048.
- Czerkawski, J. W., Breckenridge, G. (1977). Design and development of a long-term rumen simulation technique (Rusitec). *British Journal of Nutrition* 28, 371-384.
- Dehority, B. A. (2003). *Rumen microbiology*. Nottingham University Press, Nottingham.
- Dehority, B.A., Tirabasso, P.A. (1989). Factors affecting the migration and sequestration of rumen protozoa in the family Isotrichidae. *J Gen Microbiol* 135, 539-548.
- Dehority, B. A., Mattos, W. R. S. (1978). Diurnal changes and effect of ration on concentrations of the rumen ciliate *Charon ventriculi*. *Appl. Environ. Microbiol.* 36, 953-958.
- Dewhurst, R. J. (2013). Milk production from silage: Comparison of grass, legume and maize silages and their mixtures. *Agricultural and Food Science* 22, 57-69.
- Dewhurst, R. J., Wadhwa, D., Borgida, L. P., Fisher, W. J. (2001). Rumen acid production from dairy feeds. 1. Effects on feed intake and milk production of dairy cows offered grass or corn silages. *J. Dairy Sci.* 84, 2721-2729.
- Dijkstra, J., Ellis, J. L., Kebreab, E., Strathe, A. B., López, S., France, J., Bannink, A. (2012). Ruminal pH regulation and nutritional consequences of low pH. *Anim. Feed Sci. Technol.* 172, 22-33.
- Doreau, M., van der Werf, H. M. G., Micol, D., Dubroeuq, H., Agabriel, J., Rochette, Y., Martin, C. (2011). Enteric methane production and greenhouse gases balance of diets differing in concentrate in the fattening phase of a beef production system. *J. Anim. Sci.* 89, 2518-2528.
- Edwards, J. E., Huws, S. A., Kim, E. J., Kingston-Smith, A. H. (2008). Erratum: Characterization of the dynamics of initial bacterial colonization of nonconserved forage in the bovine rumen (FEMS Microbiology Ecology (2007) 62, (323-335)). *FEMS Microbiol. Ecol.* 63, 141-142.
- Firkins, J. L., Yu, Z. (2015). Ruminant nutrition symposium: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J. Anim. Sci.* 93, 1450-1470.
- Fogel, G. B., Collins, C. R., Li, J., Brunk, C. F. (1999). Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. *Microb. Ecol.* 38, 93-113.
- Friedrich, M. W. (2005). Methyl-coenzyme M reductase genes: Unique functional markers for methanogenic and anaerobic methane-oxidizing Archaea. *Methods Enzymol.* 397, 428-442.

- Ghaffari, M. H., Durmic, Z., Real, D., Vercoe, P., Smith, G., Oldham, C. (2015). Furanocoumarins in teder do not affect ruminal fermentation in continuous culture. *Animal Production Science* 55, 544-550.
- Givens, D. I., Rulquin, H. (2004). Utilisation by ruminants of nitrogen compounds in silage-based diets. *Anim. Feed Sci. Technol.* 114, 1-18.
- Gizzi, G., Zanchi, R., Sciaraffia, F. (1998). Comparison of microbiological and fermentation parameters obtained with an improved rumen *in vitro* technique with those obtained *in vivo*. *Anim. Feed Sci. Technol.* 73, 291-305.
- Griswold, K. E., Hoover, W. H., Miller, T. K., Thayne, W. V. (1996). Effect of form of nitrogen on growth of ruminal microbes in continuous culture. *J. Anim. Sci.* 74, 483-491.
- Ha, J. K., Kennelly, J. J., Berzins, R. (1986). Effect of dietary nitrogen source on microbial protein synthesis, dietary protein degradation and nutrient digestion in steers. *Anim. Feed Sci. Technol.* 14, 117-126.
- Hackmann, T. J., Firkins, J. L. (2015). Maximizing efficiency of rumen microbial protein production. *Front. Microbiol.* 6.
- Hannah, S. M., Stern, M. D., Ehle, F. R. (1986). Evaluation of a dual flow continuous culture system for estimating bacterial fermentation *in vivo* of mixed diets containing various soya bean products. *Anim. Feed Sci. Technol.* 16, 51-62.
- Hassanat, F., Gervais, R., Julien, C., Massé, D. I., Lettat, A., Chouinard, P. Y., Petit, H. V., Benchaar, C. (2013). Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *J. Dairy Sci.* 96, 4553-4567.
- Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., Waghorn, G. C., Janssen, P. H. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS ONE* 8.
- Hildebrand, B., Boguhn, J., Rodehutsord, M. (2011a). Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. *Animal* 5, 537-546.
- Hildebrand, B., Boguhn, J., Rodehutsord, M. (2011b). Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation *in vitro*. *Animal* 5, 528-536.
- Hildebrand, B., Boguhn, J., Rodehutsord, M. (2011c). Investigations on the effect of forage source, grinding, and urea supplementation on ruminal fermentation and microbial protein flow in a semi-continuous rumen simulation system. *Arch. Anim. Nutr.* 65, 402-414.
- Hobson, P. N. (1997). Introduction. In: Hobson, P. N., Stewart, C. S. (Eds.) *The rumen microbial ecosystem*. Second Edition. Blackie Academic & Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 1-9.
- Hobson, P. N., McDougall, E. I., Summers, R., (1968). The nitrogen sources of *Bacteroides amylophilus*. *J. Gen. Microbiol.* 50.

- Hook, S. E., Dijkstra, J., Wright, A. -D. G., McBride, B. W., France, J. (2012). Modeling the distribution of ciliate protozoa in the reticulo-rumen using linear programming. *J. Dairy Sci.* 95, 255-265.
- Hook, S. E., Steele, M. A., Northwood, K. S., Wright, A. -D. G., McBride, B. W. (2011). Impact of high-concentrate feeding and low ruminal pH on Methanogens and Protozoa in the rumen of dairy cows. *Microb. Ecol.* 62, 94-105.
- Hoover, W. H., Crooker, B. A., Sniffen, C. J. (1976). Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* 43, 528-534.
- Hristov, A. N., Lee, C., Hristova, R., Huhtanen, P., Firkins, J. L. (2012). A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. *J. Dairy Sci.* 95, 5299-5307.
- Huws, S. A., Lee, M. R. F., Muetzel, S. M., Scott, M. B., Wallace, R. J., Scollan, N. D. (2010). Forage type and fish oil cause shifts in rumen bacterial diversity. *FEMS Microbiol. Ecol.* 73, 396-407.
- Janssen, P. H., Kirs, M. (2008). Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74, 3619-3625.
- Jouany, J. (1996). Effect of rumen protozoa on nitrogen utilization by ruminants. *The Journal of Nutrition* 126, 1335S-1346S.
- Karsli, M. A., Russell, J. R. (2002). Effects of source and concentrations of nitrogen and carbohydrate on ruminal microbial protein synthesis. *Turk J Vet Anim Sci* 26, 201-207.
- Klappenbach, J. A., Saxman, P. R., Cole, J. R., Schmidt, T. M. (2001). Rrndb: The ribosomal RNA operon copy number database. *Nucleic Acids Res.* 29, 181-184.
- Klevenhusen, F., Deckardt, K., Sizmaz, Ö., Wimmer, S., Muro-Reyes, A., Khiaosa-Ard, R., Chizzola, R., Zebeli, Q. (2015). Effects of black seed oil and *Ferula elaeochytris* supplementation on ruminal fermentation as tested in vitro with the rumen simulation technique (Rusitec). *Ani. Prod. Sci.* 55, 736-744.
- Koike, S., Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* 204, 361-366.
- Koike, S., Pan, J., Kobayashi, Y., Tanaka, K. (2003). Kinetics of in sacco fiber-attachment of representative ruminal cellulolytic bacteria monitored by competitive PCR. *J. Dairy Sci.* 86, 1429-1435.
- Koike, S., Yabuki, H., Kobayashi, Y. (2014). Interaction of rumen bacteria as assumed by colonization patterns on untreated and alkali-treated rice straw. *Anim Sci J* 85, 524-531.
- Kong, Y., Teather, R., Forster, R. (2010). Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol. Ecol.* 74, 612-622.

- Kozakai, K., Nakamura, T., Kobayashi, Y., Tanigawa, T., Osaka, I., Kawamoto, S., Hara, S. (2007). Effect of mechanical processing of corn silage on in vitro ruminal fermentation, and in situ bacterial colonization and dry matter degradation. *Can. J. Anim. Sci.* 87, 259-267.
- Krätzer, C., Carini, P., Hovey, R., Deppenmeier, U. (2009). Transcriptional profiling of methyltransferase genes during growth of *Methanosarcina mazei* on trimethylamine. *J. Bacteriol.* 191, 5108-5115.
- Lee, M. R. F., Cabiddu, A., Hou, F., Niderkorn, V., Kim, E. J., Fychan, R., Scollan, N. D. (2011). In vitro rumen simulated (RUSITEC) metabolism of freshly cut or wilted grasses with contrasting polyphenol oxidase activities. *Grass Forage Sci.* 66, 196-205.
- Lerma, C., Rich, P. J., Ju, G. C., Yang, W. -J., Hanson, A. D., Rhodes, D. (1991). Betaine deficiency in maize: Complementation tests and metabolic basis. *Plant Physiol.* 95, 1113-1119.
- Lettat, A., Hassanat, F., Benchaar, C. (2013). Corn silage in dairy cow diets to reduce ruminal methanogenesis: Effects on the rumen metabolically active microbial communities. *J. Dairy Sci.* 96, 5237-5248.
- Ling, J. R., Armstead, I. P. (1995). The in vitro uptake and metabolism of peptides and amino acids by five species of rumen bacteria. *J. Appl. Bacteriol.* 78, 116-124.
- López, S. (2005). In vitro and in situ techniques for estimating digestibility. In: Dijkstra, J., Forbes, J. M., France, J. (Eds.) *Quantitative aspects of ruminant digestion and metabolism*. CABI Publishing, pp. 87-121.
- Ludwig, W., Schleifer, K. H. (1994). Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* 15, 155-173.
- Mackie, R. I., Gilchrist, F. M. C., Robberts, A. M., Hannah, P. E., Schwartz, H. M. (1978). Microbiological and chemical changes in the rumen during the stepwise adaptation of sheep to high concentrate diets. *J. Agric. Sci.* 90, 241-254.
- Mackie, R. I., McSweeney, C. S., Aminov, R. I. (2001). Rumen. In: Anonymous ELS. John Wiley & Sons, Ltd, Chichester.
- Martínez, M. E., Ranilla, M. J., Ramos, S., Tejido, M. L., Carro, M. D. (2011). Evolution of fermentation parameters in Rusitec fermenters operated at different dilution rates and concentrate retention times. Zaragoza : CIHEAM / CSIC / Universidad De León / FAO Options Méditerranéennes : Série A. Séminaires Méditerranéens 99, 121-126.
- Martínez, M. E., Ranilla, M. J., Tejido, M. L., Ramos, S., Carro, M. D. (2010a). Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. *J. Dairy Sci.* 93, 3684-3698.
- Martínez, M. E., Ranilla, M. J., Tejido, M. L., Saro, C., Carro, M. D. (2010b). Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. II. Protozoa population and diversity of bacterial communities. *J. Dairy Sci.* 93, 3699-3712.

- Martínez, M. E., Ranilla, M. J., Tejido, M. L., Saro, C., Carro, M. D. (2010c). The effect of the diet fed to donor sheep on in vitro methane production and ruminal fermentation of diets of variable composition. *Anim. Feed Sci. Technol.* 158, 126-135.
- Matsui, H., Ogata, K., Tajima, K., Nakamura, M., Nagamine, T., Aminov, R. I., Benno, Y. (2000). Phenotypic characterization of polysaccharidases produced by four *Prevotella* type strains. *Curr. Microbiol.* 41, 45-49.
- McAllister, T. A., Cheng, K. -J. (1996). Microbial strategies in the ruminal digestion of cereal grains. *Anim. Feed Sci. Technol.* 62, 29-36.
- McDougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of shepp's saliva. *Biochem J.* 43, 99-109.
- Mitchell, A. D., Chappell, A., Knox, K. L. (1979). Metabolism of betaine in the ruminant. *J. Anim. Sci.* 49, 764-774.
- Miyazaki, K., Martin, J.C., Marinsek-Logar, R., Flint, H. J. (1997). Degradation and utilization of xylans by the rumen anaerobe *Prevotella bryantii* (formerly *P. ruminicola* subsp. *brevis*) B14. *Anaerobe* 3, 373-381.
- Monteils, V., Rey, M., Silberberg, M., Cauquil, L., Combes, S. (2012). Modification of activities of the ruminal ecosystem and its bacterial and protozoan composition during repeated dietary changes in cows1. *J. Anim. Sci.* 90, 4431-4440.
- Narvaez, N., Wang, Y., Xu, Z., Alexander, T., Garden, S., McAllister, T. (2013). Effects of hop varieties on ruminal fermentation and bacterial community in an artificial rumen (rusitec). *J. Sci. Food Agric.* 93, 45-52.
- Neill, A. R., Grime, D. W., Dawson, R. M. C. (1978). Conversion of choline methyl groups through trimethylamine into methane in the rumen. *Biochem. J.* 170, 529-535.
- Newbold, C. J., De la Fuente, G., Belanche, A., Ramos-Morales, E., McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Front. Microbiol.* 6.
- Ogata, K., Aminov, R. I., Nagamine, T., Sugiura, M., Tajima, K., Mitsumori, M., Sekizaki, T., Kudo, H., Minato, H., Benno, Y. (1997). Construction of a *Fibrobacter succinogenes* genomic map and demonstration of diversity at the genomic level. *CURR. MICROBIOL.* 35, 22-27.
- Olubobokun, J. A., Craig, W. M., Nipper, W. A. (1988). Characteristics of protozoal and bacterial fractions from microorganisms associated with ruminal fluid or particles. *J. Anim. Sci.* 66, 2701-2710.
- O'Mara, F. P., Fitzgerald, J. J., Murphy, J. J., Rath, M. (1998). The effect on milk production of replacing grass silage with maize silage in the diet of dairy cows. *Livest. Prod. Sci.* 55, 79-87.
- Orpin, C. G., Joblin, K. N. (1997). The rumen anaerobic fungi. In: Hobson, P.N., Stewart, C.S. (Eds.) *The rumen microbial ecosystem*. Second Edition, Blackie Academic & Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 140-195.
- Owens, D., McGee, M., Boland, T., O'Kiely, P. (2009). Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat. *J. Anim. Sci.* 87, 658-668.

- Paster, B. J., Russell, J. B., Yang, C. M. J., Chow, J. M., Woese, C. R., Tanner, R. (1993). Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. *Int. J. Syst. Bacteriol.* 43, 107-110.
- Patra, A. K., Yu, Z. (2013). Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen. *Appl. Microbiol. Biotechnol.*, 1-9.
- Peterka, M., Avguštin, G. (2001). Ribosomal RNA genes from *Prevotella bryantii*: Organization and heterogeneity. *Folia Microbiol.* 46, 67-70.
- Petri, R. M., Forster, R. J., Yang, W., McKinnon, J. J., McAllister, T. A. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J. Appl. Microbiol.* 112, 1152-1162.
- Potter, E. L., Dehority, B. A. (1973). Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. *Appl. Microbiol.* 26, 692-698.
- Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R. M., Spang, A., Canibe, N., Højberg, O., Milinovich, G., Fragner, L., Schleper, C., Weckwerth, W., Lund, P., Schramm, A., Urich, T. (2013). Methylophilic methanogenic *Thermoplasmata* implicated in reduced methane emissions from bovine rumen. *Nature Communications* 4.
- Prevot, S., Senaud, J., Bohatier, J., Prensier, G. (1994). Variation in the composition of the ruminal bacterial microflora during the adaption phase in an artificial fermenter (Rusitec). *Zoolog Sci* 11, 871-878.
- Russell, J. B. (1985). Fermentation of cellodextrins by cellulolytic and noncellulolytic rumen bacteria. *Appl. Environ. Microbiol.* 49, 572-576.
- Russell, J. B., Strobel, H. J., Chen, G. J. (1988). Enrichment and isolation of a ruminal bacterium with a very high specific activity of ammonia production. *Appl. Environ. Microbiol.* 54, 872-877.
- Russi, J. P., Wallace, R. J., Newbold, C. J. (2002). Influence of the pattern of peptide supply on microbial activity in the rumen simulating fermenter (RUSITEC). *Br. J. Nutr.* 88, 73-80.
- Santra, A., Karim, S. A., Mishra, A. S., Chaturvedi, O. H., Prasad, R. (1998). Rumen ciliate protozoa and fibre utilization in sheep and goats. *Small Rumin Res* 30, 13-18.
- Saro, C., Ranilla, M. J., Tejido, M. L., Carro, M. D. (2014). Influence of forage type in the diet of sheep on rumen microbiota and fermentation characteristics. *Livest Sci* 160, 52-59.
- Seeling, K., Boguhn, J., Strobel, E., Dänicke, S., Valenta, H., Ueberschär, K. H., Rodehutschord, M. (2006). On the effects of *Fusarium* toxin contaminated wheat and wheat chaff on nutrient utilisation and turnover of deoxynivalenol and zearalenone in vitro (Rusitec). *Toxicol. Vitro* 20, 703-711.
- Shinkai, T., Kobayashi, Y. (2007). Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real-time PCR. *Appl. Environ. Microbiol.* 73, 1646-1652.

- Soto, R. C., Muhammed, S. A., Newbold, C. J., Stewart, C. S., Wallace, R. J. (1994). Influence of peptides, amino acids and urea on microbial activity in the rumen of sheep receiving grass hay and on the growth of rumen bacteria in vitro. *Anim. Feed Sci. Technol.* 49, 151-161.
- Staerfl, S. M., Zeitz, J. O., Kreuzer, M., Soliva, C. R. (2012). Methane conversion rate of bulls fattened on grass or maize silage as compared with the IPCC default values, and the long-term methane mitigation efficiency of adding acacia tannin, garlic, maca and lupine. *Agric. Ecosyst. Environ.* 148, 111-120.
- Stewart, C. S., Flint, H. J., Bryant, M. P. (1997). The rumen bacteria. In: Hobson, P.N., Stewart, C.S. (Eds.) *The rumen microbial ecosystem*. Second Edition. Blackie Academic & Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 10-72.
- Suen, G., Weimer, P. J., Stevenson, D. M., Aylward, F. O., Boyum, J., Deneke, J., Drinkwater, C., Ivanova, N. N., Mikhailova, N., Chertkov, O., Goodwin, L. A., Currie, C. R., Mead, D., Brumm, P. J. (2011). The complete genome sequence of fibrobacter succinogenes s85 reveals a cellulolytic and metabolic specialist. *PLoS ONE* 6.
- Sylvester, J. T., Karnati, S. K. R., Dehority, B. A., Morrison, M., Smith, G. L., St-Pierre, N. R., Firkins, J. L. (2009). Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. *J. Dairy Sci.* 92, 256-269.
- Tajima, K., Aminov, R. I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y. (2001). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* 67, 2766-2774.
- Trinci, A. P. J., Davies, D. R., Gull, K., Lawrence, M. I., Nielsen, B. B., Rickers, A., Theodorou, M. K. (1994). Anaerobic fungi in herbivorous animals. *Mycol. Res.* 98, 129-152.
- Tymensen, L. D., Beauchemin, K. A., McAllister, T. A. (2012). Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. *Microbiology (United Kingdom)* 158, 1808-1817.
- Valdez, R. E., Alvarez, F. J., Ferreiro, H. M., Lopez, F. G. J., Priego, A., Blackburn, T. H., Leng, R. A., Preston, T. R. (1977). Rumen function in cattle given sugar cane. *Trop. Anim. Prod.* 2, 260-272.
- Wallace, R. J., Onodera, R., Cotta, M. A. (1997). Metabolism of nitrogen-containing compounds. In: Hobson, P.N., Stewart, C.S. (Eds.) *The rumen microbial ecosystem*. Second Edition. Blackie Academic & Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 283-328.
- Wang, Y., Alexander, T. W., Mcallister, T. A. (2009). In vitro effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on rumen bacterial populations and fermentation. *J. Sci. Food Agric.* 89, 2252-2260.
- Warner, A. C. (1966). Diurnal changes in the concentrations of micro-organisms in the rumens of sheep fed limited diets once daily. *J. Gen. Microbiol.* 45, 213-235.
- Welkie, D. G., Stevenson, D. M., Weimer, P. J. (2010). ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. *Anaerobe* 16, 94-100.

- Whelan, S. J., Mulligan, F. J., Callan, J. J., Flynn, B., Pierce, K. M. (2013). Effect of forage source and a supplementary methionine hydroxyl analogue on rumen fermentation parameters in lactating dairy cows offered a low crude protein diet. *Anim. Feed Sci. Technol.* 183, 62-66.
- Williams, G., Coleman, G. S. (1997). The rumen protozoa. In: Hobson, P.N., Stewart, C.S. (Eds.) *The Rumen Microbial Ecosystem*. Second Edition. Blackie academic & professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 73-139.
- Wischer, G., Boguhn, J., Steingäß, H., Schollenberger, M., Rodehutschord, M. (2013). Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis in vitro. *Animal* 7, 1796-1805.
- Witzig, M., Boguhn, J., Kleinsteuber, S., Fetzer, I., Rodehutschord, M. (2010a). Effect of the corn silage to grass silage ratio and feed particle size of diets for ruminants on the ruminal Bacteroides-Prevotella community in vitro. *Anaerobe* 16, 412-419.
- Witzig, M., Boguhn, J., Kleinsteuber, S., Fetzer, I., Rodehutschord, M. (2010b). Influence of the maize silage to grass silage ratio and feed particle size of rations for ruminants on the community structure of ruminal Firmicutes in vitro. *J. Appl. Microbiol.* 109, 1998-2010.
- Witzig, M., Boguhn, J., Zeder, M., Seifert, J., Rodehutschord, M. (2015). Effect of donor animal species and their feeding on the composition of the microbial community establishing in a rumen simulation. *J. Appl. Microbiol.* 119, 33-46.
- Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* 51, 221-271.
- Wolin, M. J., Miller, T. L., Stewart, C. S. (1997). Microbe-microbe interactions. In: Hobson, P.N., Stewart, C.S. (Eds.) *The rumen microbial ecosystem*. Second Edition. Blackie Academic & Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp. 467-491.
- Yu, Z., Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36, 808-812.
- Zeitz, J. O., Guertler, P., Pfaffl, M. W., Eisenreich, R., Wiedemann, S., Schwarz, F. J. (2013). Effect of non-starch-polysaccharide-degrading enzymes as feed additive on the rumen bacterial population in non-lactating cows quantified by real-time PCR. *J. Anim. Physiol. Anim. Nutr.* 97, 1104-1113.
- Zhao, X. H., Liu, C. J., Liu, Y., Li, C. Y., Yao, J. H. (2013). Effects of replacing dietary starch with neutral detergent-soluble fibre on ruminal fermentation, microbial synthesis and populations of ruminal cellulolytic bacteria using the rumen simulation technique (RUSITEC). *J. Anim. Physiol. Anim. Nutr.* 97, 1161-1169.
- Zhou, M., Hernandez-Sanabria, E., Le, L. G. (2009). Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Appl. Environ. Microbiol.* 75, 6524-6533.
- Zhu, W., Fu, Y., Wang, B., Wang, C., Ye, J. A., Wu, Y.M., Liu, J. -X. (2013). Effects of dietary forage sources on rumen microbial protein synthesis and milk performance in early lactating dairy cows. *J. Dairy Sci.* 96, 1727-1734.



Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., Stahl, D. A. (2000). Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ. Microbiol.* 2, 632-643.



## CHAPTER 3

### INCLUDED MANUSCRIPTS



MANUSCRIPT 1:

CHANGES IN RUMEN MICROBIAL COMMUNITY COMPOSITION DURING  
ADAPTION TO AN *IN VITRO* SYSTEM AND THE IMPACT OF DIFFERENT  
FORAGES

Melanie B. Lengowski<sup>1</sup>, Karin H. R. Zuber<sup>1</sup>, Maren Witzig<sup>1</sup>, Jens Möhring<sup>2</sup>, Jeanette Boguhn<sup>1</sup>,  
Markus Rodehutscord<sup>1</sup>

<sup>1</sup>Institut für Nutztierwissenschaften, Universität Hohenheim, Stuttgart-Hohenheim, Baden-  
Württemberg, Germany; <sup>2</sup>Institut für Kulturpflanzenwissenschaften, Fachgebiet Biostatistik,  
Universität Hohenheim, Stuttgart-Hohenheim, Baden-Württemberg, Germany

Published in:

PLoS ONE (2016), 11(2): e0150115

(Doi:10.1371/journal.pone.0150115)



RESEARCH ARTICLE

# Changes in Rumen Microbial Community Composition during Adaption to an *In Vitro* System and the Impact of Different Forages

Melanie B. Lengowski<sup>1</sup>, Karin H. R. Zuber<sup>1</sup>, Maren Witzig<sup>1\*</sup>, Jens Möhring<sup>2</sup>, Jeannette Boguhn<sup>1</sup>, Markus Rodehutscord<sup>1</sup>

**1** Institut für Nutztierwissenschaften, Universität Hohenheim, Stuttgart-Hohenheim, Baden-Württemberg, Germany, **2** Institut für Kulturpflanzenwissenschaften, Fachgebiet Biostatistik, Universität Hohenheim, Stuttgart-Hohenheim, Baden-Württemberg, Germany

\* [inst450@uni-hohenheim.de](mailto:inst450@uni-hohenheim.de)



## OPEN ACCESS

**Citation:** Lengowski MB, Zuber KHR, Witzig M, Möhring J, Boguhn J, Rodehutscord M (2016) Changes in Rumen Microbial Community Composition during Adaption to an *In Vitro* System and the Impact of Different Forages. PLoS ONE 11(2): e0150115. doi:10.1371/journal.pone.0150115

**Editor:** Marie-Joelle Virolle, University Paris South, FRANCE

**Received:** October 31, 2015

**Accepted:** February 9, 2016

**Published:** February 29, 2016

**Copyright:** © 2016 Lengowski et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** This work was supported by Deutsche Forschungsgemeinschaft, Grant no. BO2960/1-2, Grant recipient: Jeannette Boguhn. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

This study examined ruminal microbial community composition alterations during initial adaption to and following incubation in a rumen simulation system (Rusitec) using grass or corn silage as substrates. Samples were collected from fermenter liquids at 0, 2, 4, 12, 24, and 48 h and from feed residues at 0, 24, and 48 h after initiation of incubation (period 1) and on day 13 (period 2). Microbial DNA was extracted and real-time qPCR was used to quantify differences in the abundance of protozoa, methanogens, total bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminobacter amylophilus*, *Prevotella bryantii*, *Selemonomonas ruminantium*, and *Clostridium aminophilum*. We found that forage source and sampling time significantly influenced the ruminal microbial community. The gene copy numbers of most microbial species (except *C. aminophilum*) decreased in period 1; however, adaption continued through period 2 for several species. The addition of fresh substrate in period 2 led to increasing copy numbers of all microbial species during the first 2–4 h in the fermenter liquid except protozoa, which showed a postprandial decrease. Corn silage enhanced the growth of *R. amylophilus* and *F. succinogenes*, and grass silage enhanced *R. albus*, *P. bryantii*, and *C. aminophilum*. No effect of forage source was detected on total bacteria, protozoa, *S. ruminantium*, or methanogens or on total gas production, although grass silage enhanced methane production. This study showed that the Rusitec provides a stable system after an adaption phase that should last longer than 48 h, and that the forage source influenced several microbial species.

## Introduction

The rumen hosts a complex microbial community comprised mainly of anaerobic bacteria, methanogens, protozoa, and fungi. These microorganisms break down feed constituents while producing primarily volatile fatty acids, microbial biomass, and gases. The composition of the microbial community in the rumen and the end products of fermentation depend on the diet

fed to the animals [1, 2]. For the evaluation of dietary effects on ruminal fermentation, microbial populations and microbial crude protein (CP) synthesis *in vitro* systems are widely used [3–5] to avoid expensive and time-consuming experiments with animals. In addition, *in vitro* systems permit the realization of a large number of treatments in sufficient replication within a relatively short period, along with the testing of higher levels of feed additives that might, in some cases, be potentially toxic to the animals [6]. Furthermore, employing *in vitro* systems allows the establishment of well-controlled environmental testing conditions, avoiding the variability inherent when utilizing individual animals [7].

One commonly used *in vitro* system is the semi-continuous rumen simulation technique (Rusitec) developed by Czerkawski and Breckenridge [7]. These authors reported similar types and quantities of fermentation products *in vitro* as those generated by the rumen of animals used as rumen content donors. Recently Martinez et al. [8, 9] compared certain characteristics of fermentation and microbial community composition in a Rusitec system presented with different concentrate to forage ratios and types of forages to those found in sheep in order to investigate how closely fermenters can mimic the dietary differences found *in vivo*. Differences between fermenters and animals were detected but the authors also reported that the Rusitec system simulated the *in vivo* fermentation more closely when high-forage rather than high-concentrate diets were used. Although Rusitec fermenters did not maintain protozoa numbers at levels found *in vivo*, sheep and fermenters showed similar total numbers of bacteria with high-forage diets. Different conditions between fermenters and animals may cause a preferential selection of certain bacterial strains *in vitro* [9]. Ziemer et al. [10] examined the adaption of the ruminal microbial community to a dual-flow continuous culture system during the first 240 h of incubation and identified a divergent microbial community at the end of the adaption phase compared to that in the inoculum. However, despite the identified changes in the microbial community composition, the model system used in this study supported a functional community structure similar to that found in the rumen. In contrast, studies on adaption of the microbial community in a Rusitec system have been restricted to the examination of fermentation characteristics [11]. Furthermore, studies on the diurnal changes of the ruminal microbial community composition in Rusitec systems are rare [12, 13]. Hence, the first objective of the present study was to investigate the changes of different microbial groups during adaption to the Rusitec system within the first two days of incubation and to study the diurnal changes of the microbial populations after adaptation using two different forages.

Forages in ruminant rations account for at least 40% of the ration and different forage sources have been shown to affect the microbial community composition differently both *in vivo* [14] and *in vitro* [9]. In Europe and North America grass silage (GS) and corn silage (CS) are the most important silages used in dairy cows and fattening cattle feeding. Owing to their different nutrient compositions, these silages have diverse effects on ruminal fermentation and the microbial community *in vivo* [15, 16] and *in vitro* [17, 18]. However, in most former studies the silages were combined with concentrates. Therefore, the second objective of this study was to investigate the effects on the ruminal microbial community and fermentation when incubating only GS or CS without concentrates.

## Materials and Methods

### Ethic statement

The cows used as donor animals for the inoculum in this study were housed at the Agricultural Experiment Station of Hohenheim University, location Meiereihof in Stuttgart (Germany), in strict accordance with the German Animal Welfare legislation. All procedures regarding



animal handling and treatments within this study were approved by the Ethical Commission of Animal Welfare of the Provincial Government of Baden- Württemberg, Germany.

## *In vitro* experiment

The *in vitro* experiment was carried out using a semi-continuous Rusitec system and followed the procedures described by Boguhn et al. [19]. Three lactating cows (Jersey;  $500 \pm 61.9$  kg of body weight and milk production of  $24.3 \pm 2.89$  l day<sup>-1</sup>) fitted with permanent rumen cannulas were used as donor animals for the inoculum. Two of the three donor cows were in mid-lactation and one in early-lactation two of them being in third and one in fourth lactation. The cows were offered hay and a total mixed ration containing CS and GS for *ad libitum* consumption. The inoculum was obtained from the solid and liquid phases of the rumen before the provision of new feed. Rumen contents from the three cows were mixed and filtered through two layers of linen cloth. Two Rusitec systems each comprising six fermenters in a water bath (39°C) were used in this study. The fermenters were filled with 800 ml of a 1:1 mixture of rumen liquid and artificial saliva [20] containing  $0.7 \text{ mmol l}^{-1} \text{ NH}_4^+$  from  $\text{NH}_4\text{Cl}$  (enriched with 10.39% <sup>15</sup>N; Campro Scientific GmbH, Berlin, Germany).

Silages with nutrient specifications as shown in Table 1 were oven-dried (24 h at 65°C) and ground through a 1 mm sieve; 15 g ground silage was used to fill individual nylon bags (pore size = 100 µm, Fa. Linker Industrie-Technik GmbH, Kassel, Germany). For each silage one fermenter was used and five experimental replicates ( $n = 5$ ) were carried out. The experimental replicates were started on five consecutive days to consider the daily variations in microbial communities that naturally occur in the rumen of the donor animals. At the beginning of each experimental run, fermenter was filled with only one bag of the corresponding silage whereas the second contained pooled rumen solids ( $60 \pm 5$  g). After 24 h, the latter was replaced by a second feedbag. On the following days, feedbags were replaced at 24-h intervals so that each bag was incubated for 48 h in total. Within each experimental run, an additional fermenter served as the blank control containing only one bag filled with pooled rumen solids for 24 h; this fermenter was run for the initial 48 h of incubation. Each experimental run lasted for 13 days. During the experiment, artificial saliva was continuously infused at a rate of approximately 590 ml day<sup>-1</sup>. Vertical movement of the feed containers inside the fermenters was achieved by an electric motor with 10 to 12 strokes min<sup>-1</sup>. The effluent was collected in 1 l bottles standing inside an ice-cold water bath. The gas produced was collected in 10 l bags (Linde

**Table 1. Chemical composition of the silages used for incubation.**

	CS <sup>a</sup>	GS <sup>b</sup>
<b>Dry matter (DM), %</b>	93.7	90.4
<b>Crude ash, % DM</b>	5.0	8.0
<b>Crude protein, % DM</b>	8.1	17.1
<b>NDF<sup>c</sup>, % DM</b>	39.8	41.3
<b>ADF<sup>d</sup>, % DM</b>	25.1	26.4
<b>ADL<sup>e</sup>, % DM</b>	2.0	1.5
<b>Starch, % DM</b>	31.6	-

<sup>a</sup>Corn silage,

<sup>b</sup>Grass silage,

<sup>c</sup>Neutral detergent fiber without residual ash after α amylase pretreatment,

<sup>d</sup>Acid detergent fiber,

<sup>e</sup>Acid detergent lignin.

doi:10.1371/journal.pone.0150115.t001

PLASTIGAS<sup>®</sup>-bags, Linde AG, Pullach, Germany) for quantification of gas production and methane concentrations as described previously [21].

## Sampling

Samples were taken within two time periods during each Rusitec run. In period 1, fermenter liquid (40 ml) was collected from the fermenters at 0, 2, 4, 12, 24, and 48 h after starting the incubation. A 30 ml subsample was stored at  $-20^{\circ}\text{C}$  for determination of the ammonium concentration and 1 ml aliquots were stored at  $-80^{\circ}\text{C}$  for microbial DNA extraction. Samples of rumen solids were obtained at the beginning of each Rusitec run and from feed residues in the bags after 24 and 48 h of incubation, and were stored at  $-80^{\circ}\text{C}$ . Starting on day 7 of incubation the total amount of effluent, gas production, methane, and feed residues were quantified on a daily basis until day 13 (period 2). A 70 ml sample of the effluent from each fermenter was collected each day and later pooled over days 7 to 13. For removal of feed particles and microbes, the effluent was centrifuged at  $27\,000 \times g$  at  $4^{\circ}\text{C}$  for 15 min using a Sorvall RC-5B Refrigerated Superspeed Centrifuge (GMI, Ramsey, Minnesota, USA). The particle-free fraction was stored at  $-20^{\circ}\text{C}$  for subsequent analysis of short-chain fatty acids (SCFA), ammonia-N, and  $^{15}\text{N}$  enrichment. The feed residues obtained from the nylon bags were dried for 24 h at  $65^{\circ}\text{C}$  and pooled over days 7 to 12 for the analysis of nutrient fractions according to the official methods in Germany [22]. To determine the microbial CP synthesis, 30 ml of fermenter liquids were collected daily from each fermenter and pooled over days 7 to 13 to obtain liquid-associated microbes (LAM) by differential centrifugation according to Brandt and Rohr [23] with modifications as described by Wischer et al. [24]. After centrifugation the microbial pellets were frozen at  $-20^{\circ}\text{C}$  until analysis for  $^{15}\text{N}$  enrichment. Solid-associated microbes were separated from feed residues on day 13 of incubation as described by Boguhn et al. [25]. Microbial pellets were stored at  $-20^{\circ}\text{C}$  for the subsequent analysis of  $^{15}\text{N}$  enrichment. Samples for microbial community analysis in period 2 were taken within the last 24 h of incubation. Fermenter liquid was collected at 0, 2, 4, 12, and 24 h after changing the feedbag on day 12. Samples from feed residues were collected at the end of each Rusitec run after 24 and 48 h of incubation. Samples for DNA extractions were stored at  $-80^{\circ}\text{C}$ .

## Chemical analyses

Feed residues from the bags were ground to pass through a sieve of 0.5 mm pore size and analyzed for dry matter by oven-drying for 4 h at  $103^{\circ}\text{C}$  (method 3.1) and crude ash by incineration at  $550^{\circ}\text{C}$  for 4 h (method 8.1). To determine CP, the nitrogen concentration was analyzed by the Kjeldahl method comprising acid digestion of the samples with sulfuric acid, steam distillation and determination of the ammonium formed by suitable titration technique. The resulting nitrogen concentration was multiplied by a 6.25 to gain the concentration of CP (method 4.1.1). The samples were analyzed for neutral detergent fiber by boiling for 1 h in a solution of disodium tetraborate, detergents and a thermally stable amylase (method 6.5.1). The acid detergent fiber was determined by boiling the samples for 1 h in sulfuric acid detergent solution (method 6.5.2). Starch (for CS only) was analyzed using a polarimetric approach after heating the samples in diluted hydrochloric acid (method 7.2.1). Methods are described in detail previously [22]. Samples of particle-free effluent and fermenter liquid were analyzed for ammonia concentration by steam distillation followed by end-point titration [24]. Concentrations of SCFA in the particle-free effluent were measured by gas chromatography as described by Geissler et al. [26] using 2-methylvaleric acid as an internal standard. Samples of silages, feed residues,  $^{15}\text{NH}_4\text{Cl}$ , freeze-dried microbial pellets, and particle-free effluent were ground finely and analyzed for  $^{15}\text{N}$  and N (only microbial pellets) using an elemental analyzer

(EA 1108; Carlo Erba Instruments, Biberach, Germany) combined with an isotope mass spectrometer (MS Finnigan MAT; Thermoquest Italia S.p.A., Milan, Italy). The microbial protein (microbial N multiplied by 6.25) from LAM was calculated as the difference between the input and output of  $^{15}\text{N}$  divided by the  $^{15}\text{N}$  concentration in LAM. Microbial protein originating from solid-associated microbes was calculated according to Hildebrand et al. [5]. Calculations of the degradation of nutrient fractions as well as for the efficiency of microbial CP synthesis were performed as described in detail elsewhere [19].

## Real-time quantitative (q)PCR

For quantification of the different microorganisms by real time qPCR, microbial DNA was extracted using the repeated bead-beating method as described by Yu and Morrison [27] with the following modifications: for cell lysis in fermenter liquids, 0.15 g of 0.1 mm and 0.05 g of 0.5 mm sterile zirconia beads were used whereas for cell lysis in feed residues, only 0.05 g of 0.05 mm sterile beads were used. Samples were homogenized using a FastPrep Instrument (MP Biomedicals, Eschwege, Germany) for 40 s at step 4. All centrifugation steps were carried out at room temperature at  $16\,000 \times g$  using a Heraeus Pico 17 centrifuge (Thermo Scientific, Braunschweig, Germany). DNA extracts were stored at  $-20^\circ\text{C}$ . The integrity of the isolated DNA was checked by agarose gel electrophoresis. The purity of the DNA extracts was assessed spectrophotometrically using a NanoDrop-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). DNA extracts showing a relatively low  $\text{OD}_{260}/\text{OD}_{230}$  ratio were additionally purified by ethanol precipitation according to Popova et al. [28]. After ethanol precipitation the ratios of  $\text{OD}_{260}/\text{OD}_{280}$  and  $\text{OD}_{260}/\text{OD}_{230}$  were on average (SD) 1.92 (0.14) and 1.70 (0.35), respectively. The DNA concentration in the extracts was measured fluorometrically using a Qubit<sup>®</sup> 2.0 Fluorometer and the Qubit<sup>™</sup> dsDNA BR Assay Kit (Invitrogen, Ltd., Paisley, UK) according to the manufacturer's protocol.

Conventional PCR was used to generate sample-derived DNA standards for each real-time qPCR assay. For this purpose, a composite DNA sample was prepared by pooling an equal amount of all DNA extracts. The primer sets that were used for the amplification of different species are listed in Table 2. PCR was performed with the iQ<sup>™</sup>5 Multicolor Real-Time PCR Detection System (Bio-Rad, München, Germany) in a total volume of 25  $\mu\text{l}$  containing 5  $\mu\text{l}$  of 5 $\times$  PCR-Mastermix (Bio & Sell, Feucht/Nürnberg, Germany), 14 ng of template DNA, and primer concentrations ranging from 300–900 nM. The amplification conditions were as follows: initial denaturation at  $94^\circ\text{C}$  for 5 min, 30 cycles of denaturation at  $94^\circ\text{C}$  (15–30 s), annealing at  $55\text{--}61^\circ\text{C}$  (30–60 s), and elongation at  $72^\circ\text{C}$  (10–120 s), with a terminal elongation step at  $72^\circ\text{C}$  for 5 min. The PCR products were separated by agarose gel electrophoresis to confirm the expected fragment length. Amplicons were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and were quantified fluorometrically as described for the DNA extracts. Gene copy numbers were calculated according to Lee et al. [29]. A tenfold serial dilution series of each PCR product with 5–6 degrees of dilution was used for generating standard curves.

Quantification of the gene copy numbers in each sample was also performed on the iQ<sup>™</sup>5 thermal cycler. Real-time qPCR assays were optimized for  $\text{MgCl}_2$  and primer concentrations as well as for annealing temperature. Reactions were carried out in a total volume of 20  $\mu\text{l}$  in Frimstar 96 well PCR-plates (Bio & Sell). The reaction mixtures contained 4  $\mu\text{l}$  of a 5 $\times$  my-Budget EvaGreenQPCR Mix II (Bio & Sell), 2.5 mM  $\text{MgCl}_2$ , 14 ng of template DNA, and primer concentrations as given in Table 2. The amplification for each sample was performed in duplicate and with the following conditions: initial denaturation at  $95^\circ\text{C}$  for 15 min, 35–45 cycles of denaturation at  $95^\circ\text{C}$  (15–35 s), annealing at  $55\text{--}61^\circ\text{C}$  (30–60 s), and elongation at  $72^\circ\text{C}$

**Table 2. Target organisms, target genes, optimized annealing temperatures ( $T_a$ ), and primer concentrations used in real-time quantitative PCR.**

Target organism	Target gene	$T_a$ (°C)	Primer conc. (nM)	Reference
Protozoa	18S rRNA	55	600	[30]
Methanogens	mcrA	60	900	[31]
Domain bacteria	16S rRNA	50	500	[32]
<i>Fibrobacter succinogenes</i>	16S rRNA	58	200	[32]
<i>Ruminococcus albus</i>	16S rRNA	55	500	[33]
<i>Ruminobacter amylophilus</i>	16S rRNA	60	500	[32]
<i>Prevotella bryantii</i>	16S rRNA	61	500	[32]
<i>Selenomonas ruminantium</i>	16S rRNA	59	500	[32]
<i>Clostridium aminophilum</i>	16S rRNA	56	400	[14, 34]

doi:10.1371/journal.pone.0150115.t002

(20–90 s), followed by a terminal elongation step at 72°C for 5 min. Standards were run in triplicate. On every plate, two standard curves were generated, one using PCR products for absolute quantification and one to determine the PCR efficiency in samples using a fivefold serial dilution series with 5 degrees of dilution from the pooled DNA sample. For each experimental run, one plate was run for the fermenter liquids and one for the feed residues. The specificity of amplification was determined by melting curve analysis. To determine the quantification cycle ( $C_q$ ), the background subtracted fluorescence data obtained from real-time qPCR were imported to the LinRegPCR quantitative PCR data analysis program (Version 2013.0; Ruijter et al., Department of Anatomy, Embryology & Physiology, Academic Center, Amsterdam, the Netherlands). Differences in  $C_q > 0.5$  between the two sample replicates led to exclusion of the sample from further data analysis. Absolute gene copy numbers in the samples were calculated by using the respective standard curves.

## Statistical analysis

Data for absolute gene copy numbers and for ammonia-N were analyzed using a mixed models approach (procedure PROC MIXED of the software package SAS; Version 9.3) considering the two treatment factors (ration and time) and including block effects according to the used design in Rusitec phase and the molecular characterization (qPCR) phase. The ration factor was split into sample classes (C), and forage sources (FS) within silage. The former separates inoculum and blank from silage, the latter distinguish between CS and GS. The time factor was split into period (P) and sampling time (ST) as observations were taken at six sampling times during two periods. An overview of the coding of variables used in the model is given in Table 3. The model in the syntax of Patterson [35] can be represented by:

$$C + FS \cdot C + P + ST \cdot P + FS \cdot C \cdot P + FS \cdot C \cdot P \cdot ST + R + WB \\ : B \cdot R + Place \cdot WB + F \cdot Place \cdot WB + P \cdot F \cdot Place \cdot WB$$

**Table 3. Overview of the coding of variables used in the statistical model<sup>a</sup>.**

Ration	Sample class	Forage source	Water bath	Sampling time	Fermenter	Place	d1
Inoculum	0	Inoculum	0	0	0	0	0
Blank	1	Blank	1 or 2	1 to 6	1 to 15	1 to 12	1
Silage	2	CS or GS <sup>b</sup>	1 or 2	1 to 6	1 to 15	1 to 12	1

<sup>a</sup>If more than one value is given, the variable can take any of the given values,

<sup>b</sup>CS: corn silage; GS: grass silage.

doi:10.1371/journal.pone.0150115.t003

where C, FS, P, and ST denote the treatment factors of sample class, forage source, period, and sampling time, respectively. WB, Place, F, P, R, and B denote the block factors of water bath, place within the water bath, fermenter, period, replicate, and block, respectively. R and B are block effects of the laboratory phase. Fixed effects are presented before the colon, and random effects are given after. Interactions are denoted by a dot between the corresponding main effects. Water bath and replicate effects are assumed to be random, but are taken as fixed because of the low number of values. As the ration inoculum is not included in the Rusitec run, no effects from block factors arising from the Rusitec phase were fitted. A dummy variable was used to eliminate these effects, but is dropped from the model description to simplify the presentation. We accounted for temporal correlations due to repeated measurements from the same fermenter by fitting either a constant covariance over time or an autoregressive model for temporal effects if the latter increased the model fit. Heterogeneous error variance for the sample classes was fitted using independent or autoregressive [36] error structures. The data were logarithmic (concentration of ammonia-N; gene copy numbers in feed residues: Total bacteria, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Prevotella bryantii*; gene copy numbers in fermenter liquids: *P. bryantii*, *Ruminobacter amylophilus*) or square root (remaining data on gene copy numbers) transformed to reach normality distributed error with homogeneous variances within a sample class. A multiple t-test for treatment comparisons was used only when the F-test was significant.

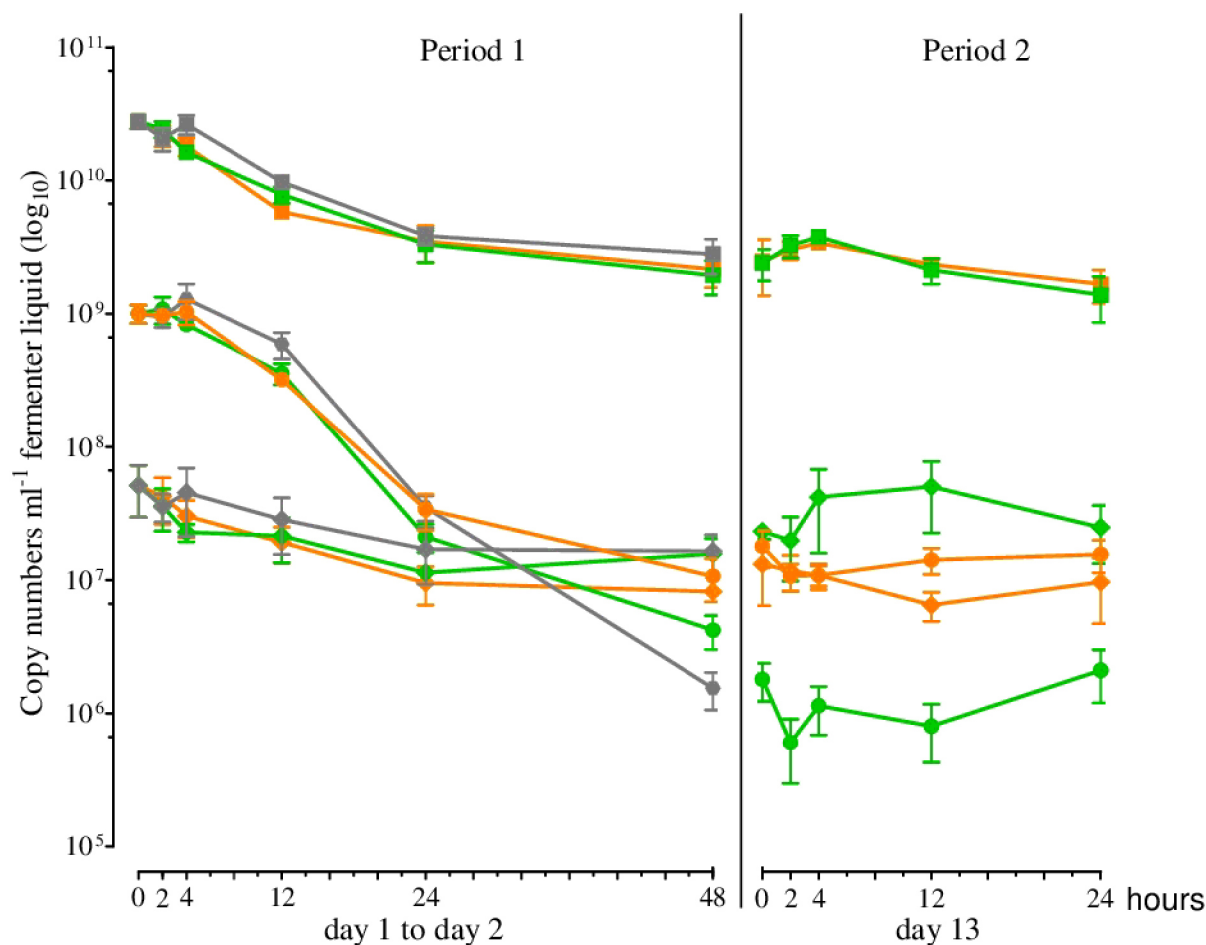
Fermentation data were analyzed by a mixed model incorporating silage, replicate, and water bath as fixed effects and fermenter as a random effect using the procedure PROC MIXED of the software package SAS (Version 9.3). Within the third experimental run, the collected total gas amount was much less compared to that obtained from the other runs; thus, the data for gas and methane concentration were omitted from the statistical analysis.

## Results

The results of real-time qPCR for the fermenter liquids are shown in Figs 1–3 and those of the solid rumen phase and feed residues in Figs 4–6 and Table 4. They are expressed as 18S rRNA, *mcrA*, and 16S rRNA gene copy numbers ml<sup>-1</sup> fermenter liquid or g<sup>-1</sup> solid rumen phase and feed residues, respectively.

### Effects of period and sampling time on the microbial populations in fermenter liquids

Within the first hours of incubation (period 1), the copy numbers of the 18S rRNA, *mcrA*, and 16S rRNA genes of all microbial species examined decreased irrespective of the incubated silage. The lowest numbers for the methanogens and for *R. albus*, *F. succinogenes*, *Selenomonas ruminantium*, and *P. bryantii* were seen after 24 h of incubation, whereas the minimum for *Clostridium aminophilum* was observed after 12 h and for protozoa and total bacteria after 48 h (Figs 1–3). No significant differences in the gene copy numbers of total bacteria, protozoa, methanogens, and *F. succinogenes* were observed between 24 and 48 h of incubation (Figs 1 and 2) but significantly higher abundances of *R. albus*, *S. ruminantium*, *P. bryantii*, and *C. aminophilum* (Figs 2 and 3) were observed after 48 h. The number of *R. amylophilus* decreased until 48 h for GS, while the minimum was found at 24 h for CS and after 48 h, the numbers returned to the level of the initial inoculum (Fig 3). No significant differences were observed between the silages and the blank control for the methanogens, protozoa, total bacteria, *R. albus*, and *S. ruminantium* (Figs 1–3). The numbers of *F. succinogenes*, *P. bryantii*, and *C. aminophilum* were significantly lower after 48 h in the blank controls compared either silage (Figs 2 and 3), whereas the numbers of *R. amylophilus* in the controls showed no significant



#### ANOVA *P* value

Effects	Protozoa	Methanogens	Domain bacteria
Period	***	0.69	**
Forage source	0.72	0.34	0.55
Forage source×period	0.18	0.26	0.86
Sampling time×period	***	***	***
Forage source×sampling time×period	0.88	0.48	0.90

**Fig 1.** 18S rRNA, *mcrA*, and 16S rRNA gene copy numbers in the fermenter liquid for protozoa (●), methanogens (◆), and total bacteria (■) at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage; gray: blank;  $n \geq 2$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ).

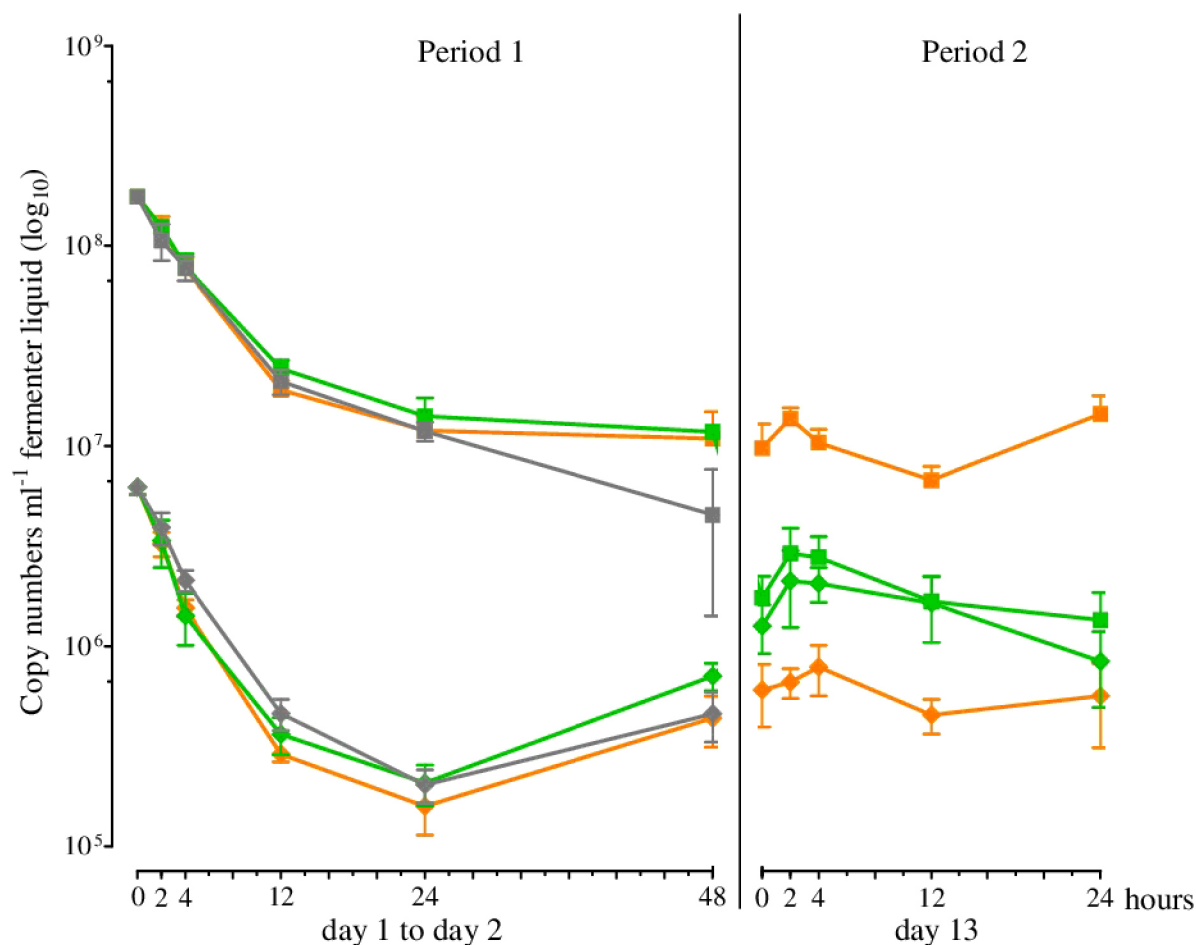
doi:10.1371/journal.pone.0150115.g001

difference compared to GS, and lower numbers were found after 48 h of incubation compared to CS (Fig 3).

At the first sampling in period 2 (0 h) the gene copy numbers of the protozoa, methanogens, total bacteria, *R. albus*, *S. ruminantium*, and *C. aminophilum* were at a similar level as those observed after 48 h of incubation in period 1 (Figs 1–3). In contrast, the numbers of *F. succinogenes* and *P. bryantii* were significantly lower in period 2 than in period 1 (Figs 2 and 3). No data was obtained for *R. amylophilus* in period 2 (Fig 3) as only nonspecific products were detected.

After the addition of a fresh feedbag at the beginning of period 2, increasing gene copy numbers in the fermenter liquids for all of the prokaryotes investigated were observed. Irrespective



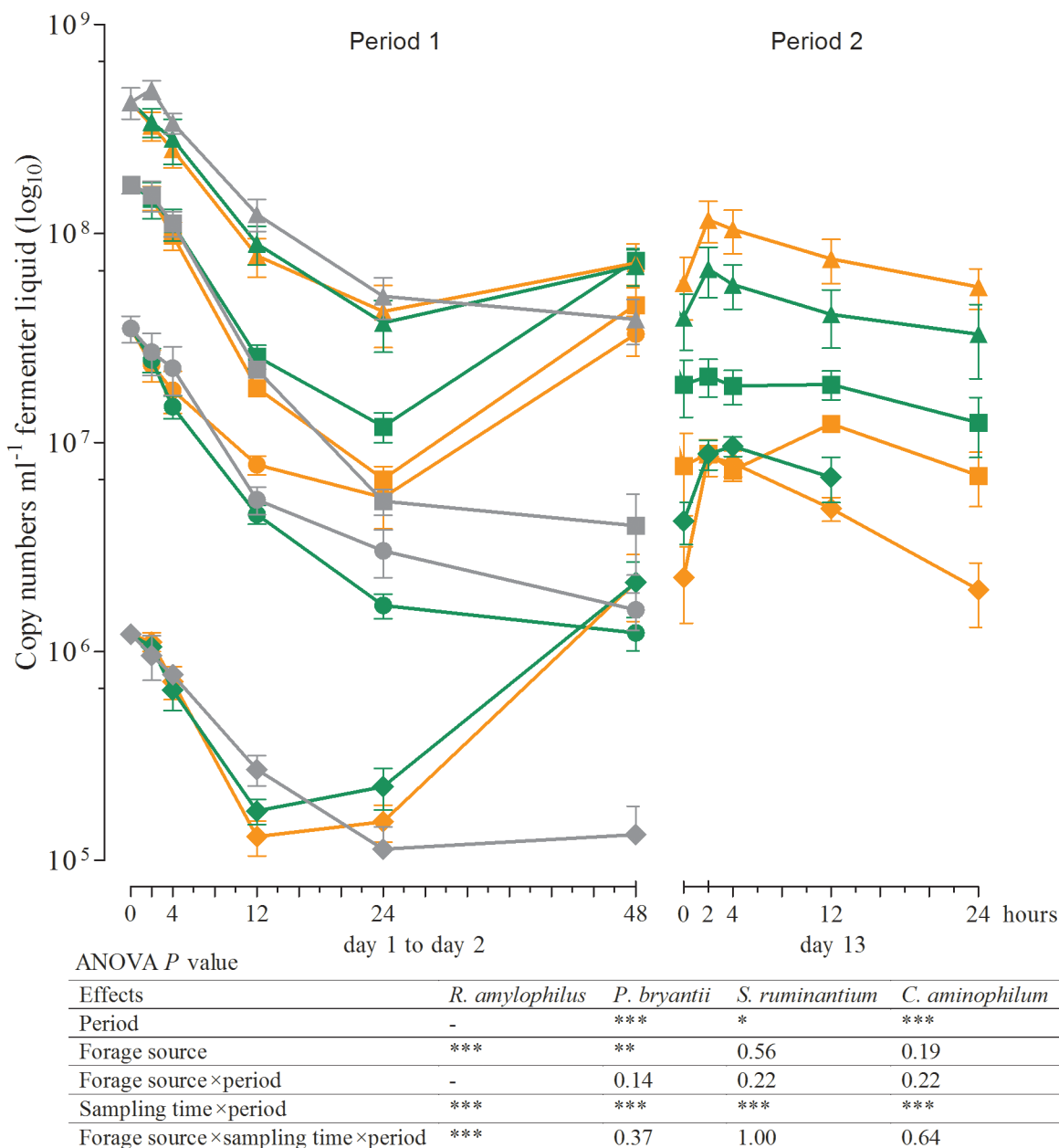
ANOVA *P* value

Effects	<i>F. succinogenes</i>	<i>R. albus</i>
Period	*	0.85
Forage source	0.15	*
Forage source×period	0.09	0.09
Sampling time×period	***	***
Forage source×sampling time×period	0.67	0.81

**Fig 2.** 16S rRNA gene copy numbers in the fermenter liquid for *Fibrobacter succinogenes* (■) and *Ruminococcus albus* (◆) at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage; gray: blank;  $n \geq 4$ ; \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ ).

doi:10.1371/journal.pone.0150115.g002

of the incubated silage, the numbers of *F. succinogenes* (Fig 2), *S. ruminantium*, and *C. aminophilum* (Fig 3) increased significantly between 0 and 2 h after feeding, whereas the gene copy numbers of the methanogens, total bacteria (Fig 1), *R. albus* (Fig 2), and *P. bryantii* (Fig 3) did not differ between sampling times. The protozoa numbers were lowest at 2 h after feedbag substitution (Fig 1); thereafter, the numbers increased up to 24 h after feeding to the levels found at the beginning of period 2. No data was obtained for *C. aminophilum* plus GS for the 24 h post-feeding sampling time, as insufficient template DNA was available for amplification (Fig 3). Irrespective of the silage used for incubation, at 24 h the numbers of the remaining microbial species examined were at the same level as at the beginning of period 2 (Figs 1–3).



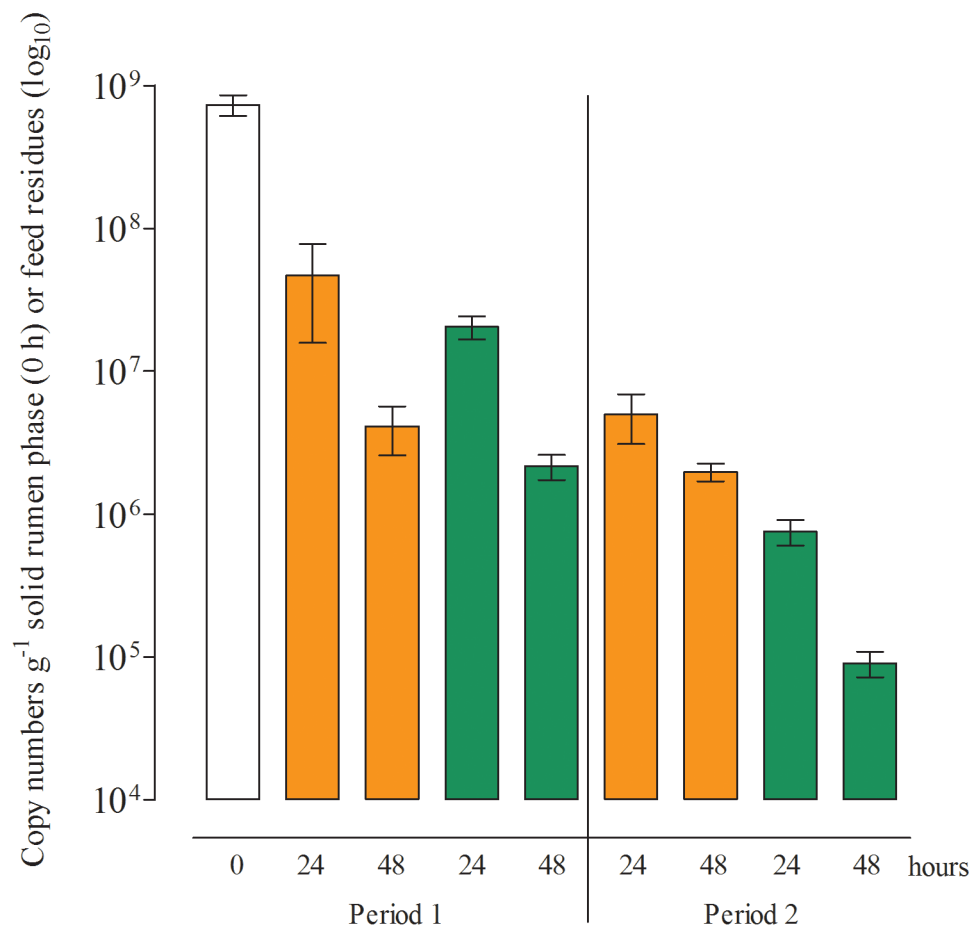
**Fig 3.** 16S rRNA gene copy numbers in the fermenter liquid for *Ruminobacter amylophilus* (●), *Prevotella bryantii* (■), *Selenomonas ruminantium* (▲), and *Clostridium aminophilum* (◆) at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage; gray: blank;  $n \geq 3$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ). *R. amylophilus* could not be detected in period 2. Owing to insufficient template DNA for amplification, no data are available on the 24 h sampling time in period 2 for *C. aminophilum* and GS.

doi:10.1371/journal.pone.0150115.g003

## Effects of period and sampling time on the microbial populations in feed residues

No effect of period or sampling time was observed for the numbers of particle-associated methanogens, total bacteria, *S. ruminantium*, *R. albus* (Table 4), and *R. amylophilus* (Fig 5) in the feed residues. The number of particle-associated protozoa significantly decreased within





	Forage source	Period	Forage source × period	Sampling time × period
ANOVA	0.40	**	0.99	**
<i>P</i> value				

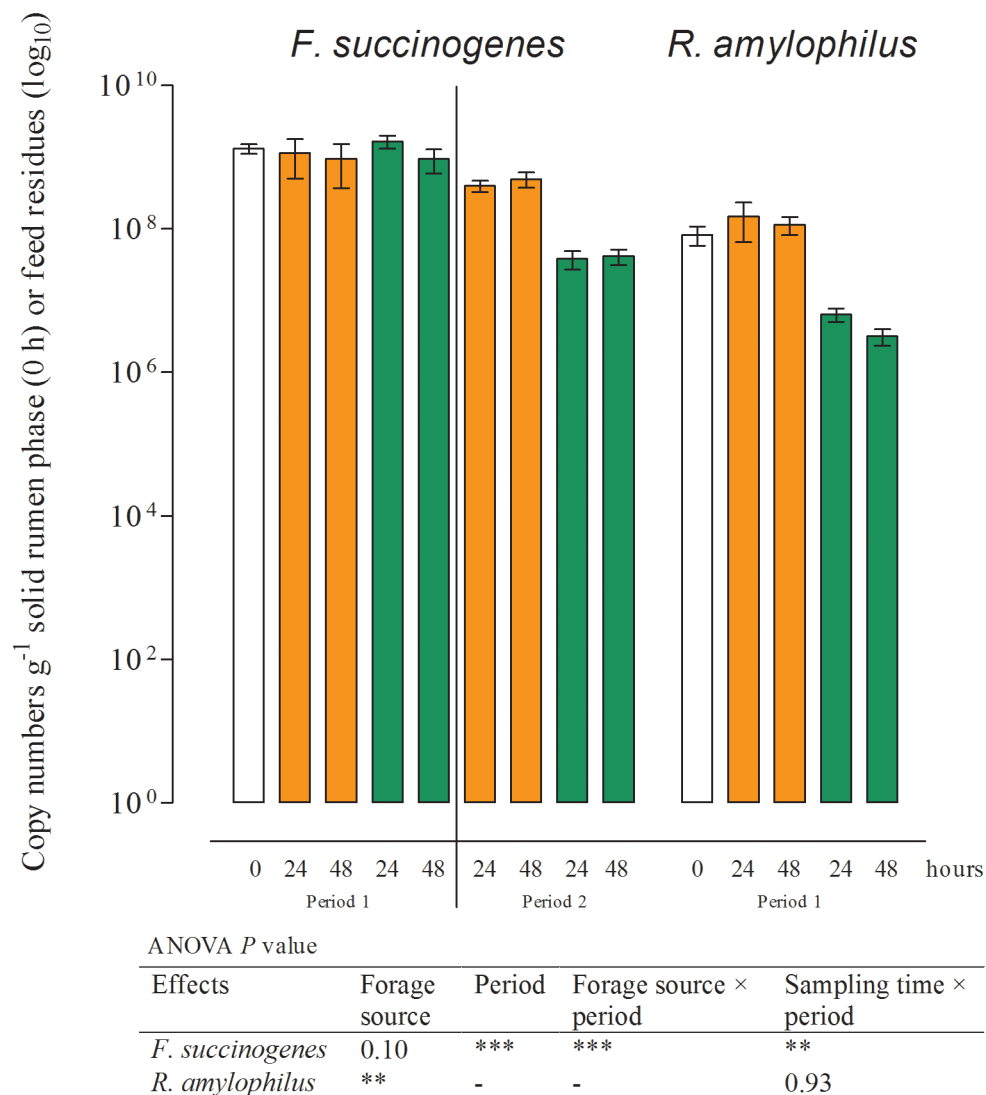
**Fig 4. 18S rRNA gene copy numbers determined in the solid rumen phase (0 h; white) and feed residues for protozoa at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage;  $n = 5$ ;  $**P \leq 0.01$ ).**

doi:10.1371/journal.pone.0150115.g004

the first 48 h of incubation (Fig 4). Similar results were found for *F. succinogenes* (Fig 5). Copy numbers for *P. bryantii* in period 1 were the lowest in the solid phase of the rumen (0 h) and highest in the feed residues at 24 h after the initiation of incubation (Fig 6). In period 2, the numbers of *P. bryantii* were significantly lower than those in period 1, but a decrease between the 24 and 48 h sampling times was also observed. The numbers of *C. aminophilum* in the solid rumen phase did not significantly differ between sampling times, but a significantly lower number of this species was observed in period 2 (Fig 6).

### Effects of forage source on the microbial populations

The numbers of protozoa, methanogens, total bacteria, and *S. ruminantium* were not affected by the forage source in either the fermenter liquids or the feed residues (Figs 1 and 3; Table 4).



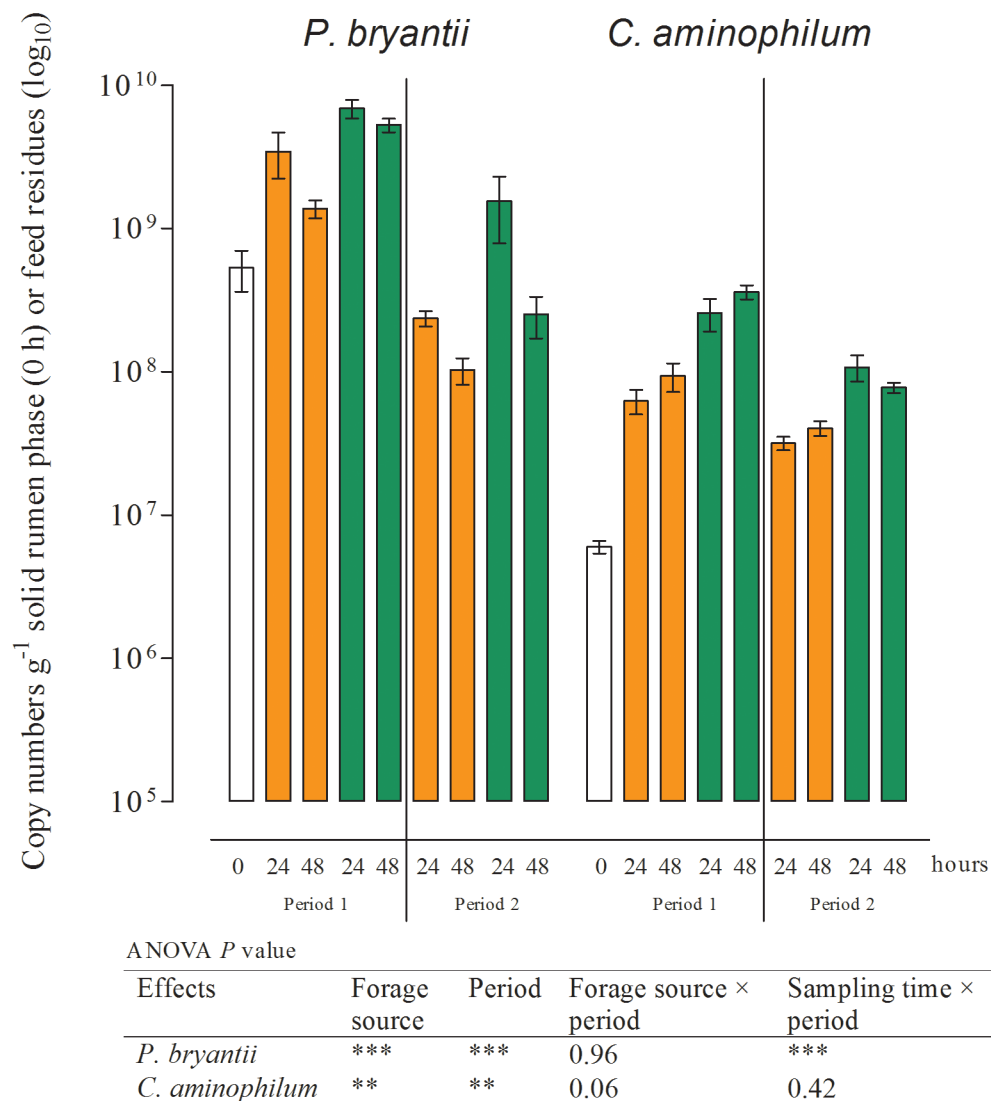
**Fig 5. 16S rRNA gene copy numbers determined in the solid rumen phase (0 h; white) and feed residues for *Fibrobacter succinogenes* and *Ruminobacter amylophilus* at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage;  $n \geq 3$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ). *R. amylophilus* could not be detected in period 2 owing to insufficient template DNA for amplification.**

doi:10.1371/journal.pone.0150115.g005

Incubation of CS resulted in higher period 2 gene copy numbers of *F. succinogenes* in both fermenter liquids as well as feed residues (Figs 2 and 5). In feed residues, higher numbers were found for *R. amylophilus* in period 1 during incubation of CS. In the fermenter liquid (Fig 3), an interaction between forage source and sampling time was detected for *R. amylophilus* gene copy numbers, wherein higher numbers were found for CS after 24 and 48 h of incubation. In contrast, the numbers of *R. albus* (Fig 2, Table 4) and *P. bryantii* (Figs 3 and 6) in both sites were increased by GS incubation.

### Effects of forage source on fermentation characteristics

The total gas production was not significantly affected by the silage used, but methane production was higher upon incubation of GS (Table 5). The degradation of organic matter



**Fig 6. 16S rRNA gene copy numbers determined in the solid rumen phase (0 h; white) and feed residues for *Prevotella bryantii* and *Clostridium aminophilum* at different times of incubation (Mean, SEM; green: grass silage; orange: corn silage;  $n \geq 4$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ).**

doi:10.1371/journal.pone.0150115.g006

and fiber fractions as well as the production of propionate and isobutyrate were also significantly higher for GS than for CS. Although the total SCFA, acetate, isovalerate, and valerate levels were not significantly affected by the silage used, the production of butyrate and the ratio of acetate to propionate were significantly higher for CS than for GS. CP degradation was similar between both silages but the amount of ammonia-N in the effluents and the efficiency of microbial CP synthesis were higher after incubation of GS. The ammonia-N in the fermenter liquids increased within the first 24 h of incubation irrespective of the forage source (Fig 7); however, after 48 h of incubation, the amount of ammonia-N was significantly higher for GS than for CS. The results from the blank controls were similar to those obtained for CS.

Table 4. McrA and 16S rRNA gene copy numbers of methanogens, total bacteria, *Ruminococcus albus*, and *Selenomonas ruminantium*.

Microbial group	Forage source	Period 1			Period 2		p- value			
		0 h	24 h	48 h	24 h	48 h	Forage source	Period	Forage source x period	Sampling time x period
Methanogens	CS <sup>a</sup>	0.96 (0.20)	0.43 (0.24)	2.67 (1.74)	0.15 (0.06)	0.36 (0.22)	n.s.	n.s.	n.s.	n.s.
	GS <sup>b</sup>		0.40 (0.12)	0.75 (0.27)	0.12 (0.05)	0.06 (0.02)				
Total bacteria	CS	562 (166)	200 (159)	256 (72.4)	362 (158)	289 (122)	n.s.	n.s.	n.s.	n.s.
	GS		315 (133)	326 (89.2)	314 (169)	247 (62.7)				
<i>R. albus</i>	CS	0.75 (0.41)	0.26 (0.36)	0.24 (0.24)	0.39 (0.21)	0.25 (0.22)	n.s.	n.s.	n.s.	n.s.
	GS		0.73 (0.66)	0.85 (0.59)	1.00 (0.98)	0.65 (0.48)				
<i>S. ruminantium</i>	CS	25.1 (2.37)	54.2 (25.1)	70.5 (37.0)	67.9 (12.2)	39.3 (18.2)	n.s.	n.s.	n.s.	n.s.
	GS		89.1 (63.1)	43.3 (16.3)	18.8 (9.07)	12.2 (3.27)				

Data were detected by real-time quantitative PCR and expressed in copy number  $\times 10^8 \text{ g}^{-1}$ ; mean (SD);  $n \geq 4$ . Gene copy numbers were determined in the solid rumen phase (0 h) and feed residues after 24 and 48 h of incubation.

<sup>a</sup>Corn silage,

<sup>b</sup>Grass silage.

doi:10.1371/journal.pone.0150115.t004

## Discussion

### Changes of the rumen microbial community composition during initial adaption to the Rusitec system

Among the microbial species investigated in the fermenter liquids and feed residues, the highest reduction over time was detected for protozoa, which occurred as early as 24–48 h following the start of incubation (Figs 1–6, Table 4). A considerable decrease of protozoa has also been described in previous *in vitro* studies [9, 10]. Compared to the generation intervals of protozoa [37], the turnover rate of the fermenter liquids in Rusitec systems is relatively high; thus, the protozoa might simply be washed out of the system [3, 38]. Protozoa are important  $\text{H}_2$  producers that play a key role in interspecies hydrogen transfer and methane production within the rumen microbial ecosystem [39]. Methanogens, conversely, are  $\text{H}_2$  consumers often showing also a physical association to protozoa [40]. However, in the present study no further decrease in the total number of methanogens was recognized in the fermenter liquids (Fig 1) after the first 24 h of incubation and no changes were found in the feed residues (Table 4), what might be expected following protozoa loss if the positive relationship between both groups is important in this system. One reason for this finding could be that in the absence of protozoa other rumen microbes that also produce  $\text{H}_2$  might increase their activity. No further decrease was found as well for the numbers of total bacteria after the first 24 h of incubation, indicating a change of the microbial community composition at the domain level during initial adaption to the Rusitec system. The changes observed for these three microbial groups in the fermenter liquids were similar to those found in the blank fermenters that contained only the solid phase from the rumen, which thus represents the approximate feed components of the diet of the donor animal. Hence, the observed shift within the microbial community cannot be solely

**Table 5. Total gas and methane production, degradation of nutrients after 48 h of incubation, ammonia-N, and short-chain fatty acids (SCFA) in the effluent, and efficiency of microbial crude protein synthesis.**

	CS <sup>a</sup>	GS <sup>b</sup>	p-value
pH	6.81 (0.04)	6.90 (0.03)	< 0.05
Total gas (ml day <sup>-1</sup> )	858 (51.2)	815 (57.8)	n.s.
Methane (ml day <sup>-1</sup> )	77.2 (4.27)	117 (13.7)	< 0.05
Degradation (%)			
Organic matter <sup>c</sup>	44.1 (0.94)	47.2 (0.97)	< 0.05
Crude protein <sup>c</sup>	67.4 (1.85)	66.5 (1.27)	n.s.
NDF <sup>d</sup>	7.86 (1.83)	17.7 (3.71)	< 0.05
ADF <sup>e</sup>	6.51 (2.17)	22.6 (1.10)	< 0.05
SCFA (mmol day <sup>-1</sup> )			
Total	34.2 (1.34)	31.9 (2.50)	n.s.
Acetate	13.6 (0.87)	12.6 (1.03)	n.s.
Propionate	5.08 (0.71)	6.63 (0.39)	< 0.05
Isobutyrate	0.32 (0.02)	0.55 (0.06)	< 0.05
Butyrate	8.79 (0.64)	6.00 (0.50)	< 0.05
Isovalerate	3.05 (0.32)	2.49 (0.55)	n.s.
Valerate	3.39 (0.26)	3.65 (0.34)	n.s.
Acetate:propionate	2.72 (0.44)	1.90 (0.06)	< 0.05
Ammonia-N in the effluent (mmol day <sup>-1</sup> )	3.04 (0.18)	7.41 (0.81)	< 0.05
Efficiency <sup>f</sup>	144 (2.70)	234 (5.37)	< 0.05

Data are expressed as the mean (SD);  $n \geq 4$ ,

<sup>a</sup>Corn silage,

<sup>b</sup>Grass silage,

<sup>c</sup>Corrected for contribution of solid associated microbes,

<sup>d</sup>Neutral detergent fiber without residual ash after  $\alpha$ -amylase treatment,

<sup>e</sup>Acid detergent fiber,

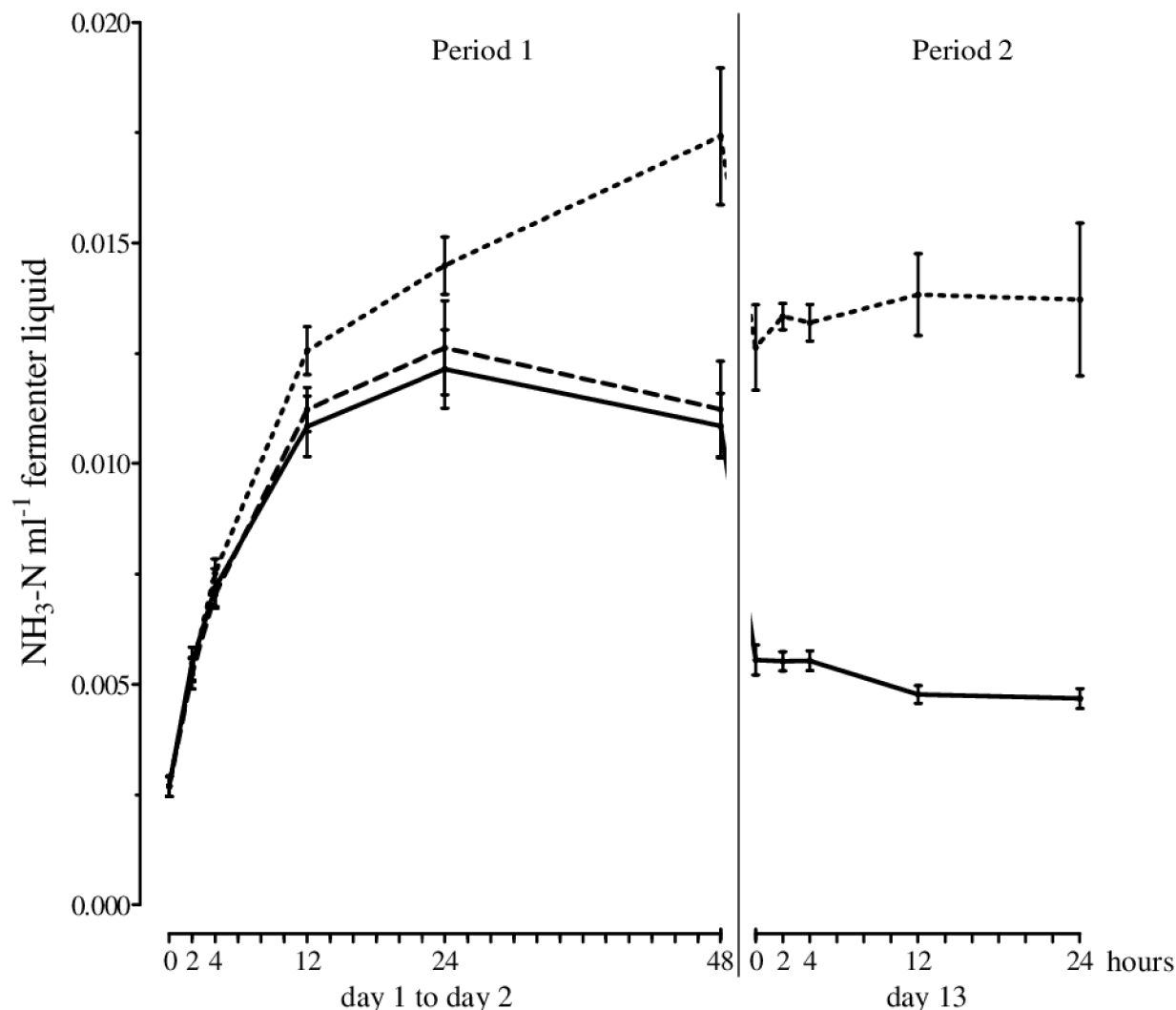
<sup>f</sup>g microbial crude protein kg<sup>-1</sup> degraded organic matter.

doi:10.1371/journal.pone.0150115.t005

linked to changes of the substrate provided for fermentation during *in vitro* incubation. Rather, the shift seemed to be a direct effect of the inoculum preparation and/or the Rusitec system itself that was still obvious after 13 days of incubation.

Changes in the microbial community composition during initial adaption to the *in vitro* system and to the forages were also seen on the species level. In fermenter liquids only the gene copy numbers of *R. albus* (Fig 2), *S. ruminantium*, *P. bryantii*, and *C. aminophilum* (Fig 3) were significantly higher after 48 h of incubation compared to those obtained at 24 h. The numbers of *C. aminophilum* increased to an even higher level than those of the inoculum, and for both *C. aminophilum* and *P. bryantii*, higher numbers were found in the feed residues compared to the solid phase (Fig 6).

On a domain level, the microbial community composition seemed to be similar at 48 h after the initiation of incubation and at the start of period 2. However, the differences in gene copy numbers found for *F. succinogenes* (Figs 2 and 5), *P. bryantii* (Figs 3 and 6), and *R. amylophilus* (Figs 3 and 5) in the fermenter liquids and feed residues, and for *C. aminophilum* (Figs 3 and 6) and protozoa (Fig 4) in the feed residues at days 2 and 13 indicated that adaption of these species to the *in vitro* system and forages was not completed by 48 h of incubation. We note that *R. amylophilus* (Figs 3 and 5) could not be quantified in period 2 because of the generation



**Fig 7. Ammonia-N (mmol in the fermenter liquid) over time for grass silage (dotted line), corn silage (solid line), and blank (dashed line; Mean, SEM;  $n \geq 4$ ). Significant differences between sampling times are described in the text.**

doi:10.1371/journal.pone.0150115.g007

of non-specific PCR products that indicated either the presence of other *Ruminobacter* strains not amplified by the primers or a substantial decrease in the number of that species, what may indicate a lack of adaption to the Rusitec system.

### Diurnal changes of the microbial populations in the Rusitec system at the end of incubation (Period 2)

The provision of fresh substrate to the fermenters via a new feedbag at the beginning of period 2 led to increasing gene copy numbers of most microbial species in the fermenter liquids within the following 2 h of incubation (Figs 1–3). Belanche et al. [12] also detected the highest numbers of total bacteria 2 h after de novo incubation of a fresh bag with ryegrass or red clover in the Rusitec system. In the present study, only the protozoa and methanogens decreased in numbers in the fermenter liquids during the first 2 h after changing the feedbag. This postprandial decrease of protozoa in the fermenter liquids might be attributed to a migration from the fermenter liquids to new feed particles [41]. After breakdown of the available nutrients,

subsequent migration back to the fermenter liquids might explain the high numbers of protozoa found 24 h after addition of the new substrate. This assumption is corroborated by the fact that in the feed residues, lower protozoa numbers were found after 48 h of incubation compared to those observed at 24 h (Fig 4). The abundance of methanogens in the fermenter liquids showed similar trends as for protozoa (Fig 1), in contrast to the situation observed following the initiation of the adaption period described previously, which might be caused by the fact that protozoa are important  $H_2$  producers [39] and that most of the ruminal methanogens use  $H_4$  and  $CO_2$  for methanogenesis [42].

In feed residues, the effect of sampling time was restricted to protozoa and *P. bryantii* (Figs 4 and 6). This is in accordance with Welkie et al. [43], who reported that the solid-associated microbial community showed less change in composition within and across feeding cycles compared to that seen for liquid-associated microbes using automated ribosomal intergenic spacer analysis (ARISA). Furthermore Craig et al. [44] reported that the level of particle-associated microbial organic matter was greatest soon after feeding. However, in the present study sampling of feed residues was only possible after 24 and 48 h of incubation; thus, possible changes in the populations of different particle-associated microbes directly after the initiation of incubation or after feeding could not be determined.

At 24 h after feed supplementation within period 2, the absolute numbers of almost all species examined were similar to those observed at the beginning of sampling on day 12, indicating that the microbial populations reached a dynamic steady state in the fermenter liquids within this *in vitro* system. In accordance with our results, Belanche et al. [12] identified similar microbial growth curves at days 10, 11, and 12 of incubation of ryegrass or red clover in the Rusitec system.

## Effects of the incubated forage source on the microbial populations and fermentation characteristics

The effect of forage source on various microbial species and the characteristics of fermentation are linked to differences in the chemical composition between silages. CS generally has a higher concentration of non-structural carbohydrates, primarily starch, and GS contains higher concentrations of CP (Table 1) and degradable fiber fractions (Table 5). Protozoa engulf and digest large numbers of bacteria and possess amylolytic as well as proteolytic [45] and cellulolytic activities [46]. This diversity in physiology might be the reason why no significant effect of forage source on the numbers of protozoa was detected in our study (Fig 4).

The forage source also did not affect the numbers of total bacteria or methanogens, which corresponds to the similar amounts of SCFAs and total gas production per day identified between the silages (Table 5). However, methane production was higher for GS compared to CS although the numbers of methanogens did not differ. One explanation for this observation might be underlying changes in methanogenic order composition, which would require that methanogenic orders with lower methanogenic activity were preferentially inhibited while those with higher methanogenic activities were enhanced [47]. It is not possible to test this hypothesis from results of the present study as detection was only performed on the group level.

Because of its cellulolytic activity, *F. succinogenes* was expected to be more abundant upon incubation of GS rather than CS. However, within period 2 we found higher numbers of this species in the fermenter liquids and in the feed residues after incubation of CS. Similar results have been reported by Lettat et al. [16], who found higher numbers of *F. succinogenes* in dairy cows fed with diets high in CS compared to those fed diets high in alfalfa silage. One explanation of this finding could be the differences in cell wall structure between  $C_3$  and  $C_4$  plants

known to affect their degradation by microorganisms [48].  $C_4$  plants as corn possess a much tougher cell wall compared to  $C_3$  plants from which GS is obtained. *F. succinogenes* is able to hydrolyze a wide variety of polysaccharides but can only utilize cellulose and its hydrolytic products for growth. Furthermore, the mechanism by which this species degrades cellulose is not completely understood but it is obvious that it stands in strong contrast to the strategies used by other cellulolytic microbes [49]. Thus, it could be possible, that *F. succinogenes* has an advantage in degrading  $C_4$  plant cell walls but this needs further research.

In the present study, the amount of fermented acid detergent fiber and CP were higher with fermentation of GS compared to that observed with CS. This is in accordance with the higher observed numbers of *R. albus* and *P. bryantii* because the former has cellulolytic activity and the latter ferments peptides and amino acids. The higher amount of ammonia-N in the effluent also confirms the assumption of higher amino acid fermentation when GS was incubated. *P. bryantii* is also involved in the degradation of hemicelluloses [50]; accordingly, a higher breakdown of structural carbohydrates such as hemicelluloses from GS was indicated by the higher neutral detergent fiber degradation and might in part be a result of the increased number of *P. bryantii*.

*R. amylophilus* requires starch or maltose as an energy source [50, 51]. Hence, higher numbers of this species found upon CS incubation compared to GS in period 1 were expected and our results are in accordance with those of Petri et al. [37], who found a higher relative abundance of *R. amylophilus* in cattle fed a diet with 49% compared to 35% starch. However, in period 2, the high supply of starch provided by CS did not lead to a further establishment of the *R. amylophilus* strains targeted by our primers, as previously discussed. Whether other strains were able to survive in the Rusitec system remains to be elucidated.

The ability of *S. ruminantium* to utilize starch [51], the products of starch hydrolysis [52], and the degradation products of cellulolytic bacteria [53] as energy sources is likely the reason for the missing effect of silage on that species.

*C. aminophilum* belongs to the class of hyper ammonia-producing bacteria (HAB). It uses only peptides and amino acids as energy sources while producing a high amount of ammonia [54]. Consequently, a higher abundance of *C. aminophilum* was expected to occur upon GS incubation. However, in the present study no differences in numbers of this organism were found between the silages. The higher amount of ammonia-N in the fermenter liquids (Fig 7) produced when GS was used could potentially not only be produced by this species but from others species involved in peptide and amino acid fermentation such as *P. bryantii*, which was shown to exhibit significantly increased numbers upon GS incubation.

Similar to the results of other *in vitro* studies [5, 19, 55] but contrary to the findings of Givens and Rulquin [56], we found that the efficiency of microbial CP synthesis was higher with incubation of GS compared to that observed with CS. This indicates that the content of available N as well as the N source could play important roles in the efficiency of microbial CP synthesis, as has been shown by several previous studies [4, 57].

The low acetate-to-propionate ratio in the current study seems to be specific for this *in vitro* system; similar ratios have been reported in previous studies [25, 58]. The low digestibility of fiber fractions might have resulted from lower cellulolytic activity due to the presence of corn starch negatively affecting microbial cellulolytic activity [18].

## Conclusion

To the best of our knowledge, this is the first study investigating the different ruminal microbial populations during initial adaption to a semi-continuous Rusitec system within the first 48 h of incubation. Our results suggest that on the domain level a stable microbial community



composition was achieved after 48 h under the given incubation conditions. However, some species showed different numbers in period 2, indicating incomplete adaptation of these species to the *in vitro* system and forage after 48 h of incubation. Our findings on protozoa confirm the results of previous studies generated with other rumen models [3, 10] that showed a substantial initial decrease of the protozoa population *in vitro*. Consequently, we suggest that *in vitro* systems are only suitable to a limited extent to investigate the protozoan population.

In addition, our data suggested that the microbial populations reached a dynamic steady state in the fermenter liquids within this *in vitro* system after an adaption phase, and that this phase should last longer than 48 h for complete adaptation of all organisms. The different chemical composition of the two silages caused a different response of the microbial populations when each was used as the forage source. In particular, the growth of *F. succinogenes*, one of the most important cellulolytic bacteria in the rumen, was favored by the incubation of CS.

## Author Contributions

Conceived and designed the experiments: MBL KHRZ MW JM JB MR. Performed the experiments: MBL KHRZ. Analyzed the data: MBL KHRZ JM. Contributed reagents/materials/analysis tools: MW JM MR. Wrote the paper: MBL KHRZ MW JM MR.

## References

1. Dewhurst RJ, Wadhwa D, Borgida LP, Fisher WJ. Rumen acid production from dairy feeds. 1. Effects on feed intake and milk production of dairy cows offered grass or corn silages. *J Dairy Sci.* 2001; 84(12):2721–2729. PMID: [11814028](#)
2. Belanche A, Doreau M, Edwards JE, Moorby JM, Pinloche E, Newbold CJ. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J Nutr.* 2012; 142(9):1684–1692. doi: [10.3945/jn.112.159574](#) PMID: [22833657](#)
3. Abe M, Kumeno F. In vitro simulation of rumen fermentation: apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. *J Anim Sci.* 1973; 36(5):941–948. PMID: [4735756](#)
4. Griswold KE, Hoover WH, Miller TK, Thayne WV. Effect of Form of Nitrogen on Growth of Ruminant Microbes in Continuous Culture. *J Anim Sci.* 1996; 74(2):483–491. PMID: [8690686](#)
5. Hildebrand B, Boguhn J, Rodehutsord M. Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. *Animal.* 2011; 5(4):537–546. doi: [10.1017/S1751731110002156](#) PMID: [22439950](#)
6. Hristov AN, Lee C, Hristova R, Huhtanen P, Firkins JL. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. *J Dairy Sci.* 2012; 95(9):5299–5307. doi: [10.3168/jds.2012-5533](#) PMID: [22916935](#)
7. Czerkawski JW, Breckenridge G. Design and development of a long-term rumen simulation technique (Rusitec). *Br J of Nutr.* 1977; 28(3):371–384.
8. Martínez ME, Ranilla MJ, Tejido ML, Ramos S, Carro MD. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. *J Dairy Sci.* 2010; 93(8):3684–3698. doi: [10.3168/jds.2009-2933](#) PMID: [20655438](#)
9. Martínez ME, Ranilla MJ, Tejido ML, Saro C, Carro MD. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. II. Protozoa population and diversity of bacterial communities. *J Dairy Sci.* 2010; 93(8):3699–3712. doi: [10.3168/jds.2009-2934](#) PMID: [20655439](#)
10. Ziemer CJ, Sharp R, Stern MD, Cotta MA, Whitehead TR, Stahl DA. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ Microbiol.* 2000; 2(6):632–643. PMID: [11214796](#)
11. Ranilla MJ, Carro MD, Ben Salem H, Morand-Fehr P, editors. Evolution of fermentation parameters in Rusitec fermenters operated at different dilution rates and concentrate retention times. Challenging strategies to promote the sheep and goat sector in the current global context.; 2011.

12. Belanche A, Lee MRF, Moorby JM, Newbold CJ. Comparison of ryegrass and red clover on the fermentation pattern, microbial community and efficiency of diet utilisation in the rumen simulation technique (Rusitec). *Anim Prod Sci*. 2013; 53(10):1052–1064.
13. Lee MRF, Cabiddu A, Hou F, Niderkorn V, Kim EJ, Fychan R, et al. In vitro rumen simulated (RUSITEC) metabolism of freshly cut or wilted grasses with contrasting polyphenol oxidase activities. *Grass Forage Sci*. 2011; 66(2):196–205.
14. Kong Y, Teather R, Forster R. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol Ecol*. 2010; 74(3):612–622. doi: [10.1111/j.1574-6941.2010.00977.x](https://doi.org/10.1111/j.1574-6941.2010.00977.x) PMID: [21044097](https://pubmed.ncbi.nlm.nih.gov/21044097/)
15. Brask M, Lund P, Hellwing ALF, Poulsen M, Weisbjerg MR. Enteric methane production, digestibility and rumen fermentation in dairy cows fed different forages with and without rapeseed fat supplementation. *Anim Feed Sci Technol*. 2013; 184(1–4):67–79.
16. Lettat A, Hassanat F, Benchaar C. Corn silage in dairy cow diets to reduce ruminal methanogenesis: Effects on the rumen metabolically active microbial communities. *J Dairy Sci*. 2013; 96(8):5237–5248. doi: [10.3168/jds.2012-6481](https://doi.org/10.3168/jds.2012-6481) PMID: [23769352](https://pubmed.ncbi.nlm.nih.gov/23769352/)
17. Witzig M, Boguhn J, Kleinstaub S, Fetzer I, Rodehutsord M. Effect of the corn silage to grass silage ratio and feed particle size of diets for ruminants on the ruminal *Bacteroides*-*Prevotella* community in vitro. *Anaerobe*. 2010; 16(4):412–419. doi: [10.1016/j.anaerobe.2010.05.002](https://doi.org/10.1016/j.anaerobe.2010.05.002) PMID: [20493960](https://pubmed.ncbi.nlm.nih.gov/20493960/)
18. Hildebrand B, Boguhn J, Rodehutsord M. Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation in vitro. *Animal*. 2011; 5(4):528–536. doi: [10.1017/S1751731110002211](https://doi.org/10.1017/S1751731110002211) PMID: [22439949](https://pubmed.ncbi.nlm.nih.gov/22439949/)
19. Boguhn J, Kluth H, Rodehutsord M. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. *J of Dairy Sci*. 2006; 89(5):1580–1591.
20. McDougall EI. Studies on ruminant saliva. 1. The composition and output of shepp's saliva. *Biochem. J* 1948; 43:99–109.
21. Wischer G, Boguhn J, Steingaß H, Schollenberger M, Rodehutsord M. Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis in vitro. *Animal*. 2013; 7(11):1796–1805. doi: [10.1017/S1751731113001481](https://doi.org/10.1017/S1751731113001481) PMID: [23915496](https://pubmed.ncbi.nlm.nih.gov/23915496/)
22. VDLUFA editor. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Vol. III. Die chemische Untersuchung von Futtermitteln. 3rd ed. Darmstadt: VDLUFA-Verlag; 2006.
23. Brandt M, Rohr K. Beiträge zur Quantifizierung der N-Umsetzung in den Vormägen von Milchkühen 1. Mitteilung: Bestimmung des Mikrobenstickstoffs im Duodenalchymus mit Hilfe von <sup>15</sup>N. *Z Tierphysiol Tierernähr Futtermittelkd*. 1981; 46:39–48.
24. Wischer G, Boguhn J, Steingaß H, Schollenberger M, Hartung K, Rodehutsord M. Effect of monensin on in vitro fermentation of silages and microbial protein synthesis. *Arch Anim Nutr*. 2013; 67(3):219–234. doi: [10.1080/1745039X.2013.793050](https://doi.org/10.1080/1745039X.2013.793050) PMID: [23679006](https://pubmed.ncbi.nlm.nih.gov/23679006/)
25. Boguhn J, Zuber T, Rodehutsord M. Effect of donor animals and their diet on in vitro nutrient degradation and microbial protein synthesis using grass and corn silages. *J Anim Physiol Anim Nutr*. 2012.
26. Geissler C, Hoffmann M, Hickel B. Ein Beitrag zur gaschromatographischen Bestimmung flüchtiger Fettsäuren. *Arch Tierernähr*. 1976; 26:123–129.
27. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques*. 2004; 36(5):808–812. PMID: [15152600](https://pubmed.ncbi.nlm.nih.gov/15152600/)
28. Popova M, Martin C, Morgavi DP. Improved protocol for high-quality co-extraction of DNA and RNA from rumen digesta. *Folia Microbiol*. 2010; 55:368–372.
29. Lee C, Lee S, Shin SG, Hwang S. Real-time PCR determination of rRNA gene copy number: absolute and relative quantification assays with *Escherichia coli*. *Appl Microbiol Biotechnol*. 2008; 78:371–376. PMID: [18074129](https://pubmed.ncbi.nlm.nih.gov/18074129/)
30. Sylvester JT, Karnati SKR, Yu Z, Morrison M, Firkins JL. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J Nutr*. 2004; 134(12):3378–3384. PMID: [15570040](https://pubmed.ncbi.nlm.nih.gov/15570040/)
31. Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol* 2007; 62(3):313–322. PMID: [17949432](https://pubmed.ncbi.nlm.nih.gov/17949432/)
32. Stevenson DM, Weimer PJ. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl Microbiol Biotechnol*. 2007; 75(1):165–174. PMID: [17235560](https://pubmed.ncbi.nlm.nih.gov/17235560/)

33. Koike S, Kobayashi Y. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol Lett*. 2001; 204(2):361–366. PMID: [11731149](#)
34. Krause DO, Russell JB. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl Environ Microbiol*. 1996; 62(3):815–821. PMID: [8975611](#)
35. Patterson HD. Analysis of series of variety trials. In: Kempton RA, Fox PN, editors. *Statistical methods for plant variety evaluation*. London: Chapman and Hall; 1997. p. 139–161.
36. Gilmour A, R., Cullis BR, Verbyla A, P. Natural and extraneous variation in the analysis of field experiments. *J Agric Biol Environ Stat*. 1997; 2:269–293.
37. Potter EL, Dehority BA. Effects of changes in feed level, starvation, and level of feed after starvation upon the concentration of rumen protozoa in the ovine. *Appl Microbiol*. 1973; 26(5):692–698. PMID: [4202706](#)
38. Muetzel S, Lawrence P, Hoffmann EM, Becker K. Evaluation of a stratified continuous rumen incubation system. *Anim Feed Sci Technol*. 2009 5/12; 151(1–2):32–43.
39. Morgavi DP, Martin C, Jouany J, Ranilla MJ. Rumen protozoa and methanogenesis: Not a simple cause-effect relationship. *Br J Nutr*. 2012; 107(3):388–397. doi: [10.1017/S0007114511002935](#) PMID: [21762544](#)
40. Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methanogenesis in ruminants. *Animal*. 2010; 4(7):1024–1036. doi: [10.1017/S1751731110000546](#) PMID: [22444607](#)
41. Valdez RE, Alvarez FJ, Ferreiro HM, Lopez FGJ, Priego A, Blackburn TH, et al. Rumen function in cattle given sugar cane. *Trop Anim Prod*. 1977; 2:260–272.
42. Janssen PH, Kirs M. Structure of the archaeal community of the rumen. *Appl Environ Microbiol*. 2008; 74(12):3619–3625. doi: [10.1128/AEM.02812-07](#) PMID: [18424540](#)
43. Welkie DG, Stevenson DM, Weimer PJ. ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. *Anaerobe*. 2010; 16(2):94–100. doi: [10.1016/j.anaerobe.2009.07.002](#) PMID: [19615457](#)
44. Craig WM, Broderick GA, Ricker DB. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J Nutr*. 1987; 117(1):56–62. PMID: [3819876](#)
45. Jouany J. Effect of Rumen Protozoa on Nitrogen Utilization by Ruminants. *J Nutr*. 1996 April 01; 126(4 Suppl):1335S–1346S. PMID: [8642481](#)
46. Williams G, Coleman GS. The rumen protozoa. In: Hobson PN, Stewart CS, editors. *The rumen microbial ecosystem*. second edition. London, Weinheim, New York, Tokyo, Melbourne, Madras: Blackie academic & professional; 1997. p. 73–139.
47. Soliva CR, Meile L, Hindrichsen IK, Kreuzer M, Machmüller A. Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. *Anaerobe*. 2004; 10(5):269–276. PMID: [16701527](#)
48. Akin DE. Histological and physical factors affecting digestibility of forages. *Agron J*. 1989; 81:17–25.
49. Suen G, Weimer PJ, Stevenson DM, Aylward FO, Boyum J, Deneke J, et al. The complete genome sequence of *Fibrobacter succinogenes* s85 reveals a cellulolytic and metabolic specialist. *PLoS ONE*. 2011; 6(4).
50. Stewart CS, Flint HJ, Bryant MP. The rumen bacteria. In: Hobson PN, Stewart CS, editors. *The rumen microbial ecosystem*. Second edition. London, Weinheim, New York, Tokyo, Melbourne, Madras: Blackie Academic & Professional; 1997. p. 10–72.
51. Cotta MA. Amylolytic activity of selected species of ruminal bacteria. *Appl Environ Microbiol*. 1988 March 01; 54(3):772–776. PMID: [2454075](#)
52. Cotta MA. Interaction of ruminal bacteria in the production and utilization of maltooligosaccharides from starch. *Appl Environ Microbiol*. 1992 January 01; 58(1):48–54. PMID: [1539992](#)
53. Russell JB. Fermentation of cellodextrins by cellulolytic and noncellulolytic rumen bacteria. *Appl Environ Microbiol*. 1985; 49(3):572–576. PMID: [3994365](#)
54. Paster BJ, Russell JB, Yang CMJ, Chow JM, Woese CR, Tanner R. Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. *Int J Syst Bacteriol*. 1993; 43(1):107–110. PMID: [8427801](#)
55. Hildebrand B, Boguhn J, Rodehutsord M. Investigations on the effect of forage source, grinding, and urea supplementation on ruminal fermentation and microbial protein flow in a semi-continuous rumen simulation system. *Arch Anim Nutr*. 2011; 65(5):402–414. PMID: [22164961](#)
56. Givens DI, Rulquin H. Utilisation by ruminants of nitrogen compounds in silage-based diets. *Anim Feed Sci Technol*. 2004; 114(1–4):1–18.

57. Carro MD, Miller EL. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). *Br J Nutr.* 1999; 82(2):149–157. PMID: [10743487](#)
58. Boguhn J, Kluth H, Bulang M, Engelhard T, Rodehutschord M. Effects of pressed beet pulp silage inclusion in maize-based rations on performance of high-yielding dairy cows and parameters of rumen fermentation. *Animal.* 2010; 4(1):30–39. doi: [10.1017/S1751731109990735](#) PMID: [22443615](#)





MANUSCRIPT 2:

EFFECTS OF SUPPLEMENTING MAIZE SILAGE WITH DIFFERENT NITROGEN  
SOURCES ON RUMINAL FERMENTATION AND MICROBIAL POPULATIONS *IN*  
*VITRO*

Maren Witzig<sup>1</sup>, Melanie B. Lengowski<sup>1</sup>, Karin H. R. Zuber<sup>1</sup>, Jens Möhring<sup>2</sup>, Markus  
Rodehutscord<sup>1</sup>

<sup>1</sup>Institut für Nutztierwissenschaften, Universität Hohenheim, Stuttgart, Germany; <sup>2</sup>Institut für  
Kulturpflanzenwissenschaften, Universität Hohenheim, Stuttgart, Germany

Submitted to:  
PLOS ONE





**Effects of supplementing maize silage with different nitrogen sources on ruminal fermentation and microbial populations *in vitro***

Maren Witzig<sup>1\*</sup>, Melanie B. Lengowski<sup>1</sup>, Karin H.R. Zuber<sup>1</sup>, Jens Möhring<sup>2</sup>, Markus Rodehutschord<sup>1</sup>

<sup>1</sup> Institut für Nutztierwissenschaften, Universität Hohenheim, Stuttgart, Germany

<sup>2</sup> Institut für Kulturpflanzenwissenschaften, Universität Hohenheim, Germany

\* Corresponding author

Email: [inst450@uni-hohenheim.de](mailto:inst450@uni-hohenheim.de)

Short title: Ruminal microbial protein synthesis

**Abstract**

Compared to grass silage (GS), maize silage (MS)-based diets seem to favor the efficiency of microbial protein synthesis (EMPS) in the rumen. Opposite findings on EMPS obtained *in vitro* have raised the question whether an inadequate supply of amino N for microbes might explain the low EMPS detected for MS *in vitro*. Thus, we examined the effect of supplementation of different N sources to MS on EMPS and microbial populations *in vitro*. GS and MS were used as substrates for *in vitro* incubation. MS was either non-supplemented or supplemented with urea, a mix of amino acids (AA), peptone, or protein to adjust the N content to that of GS. Results of degradation of organic matter and crude protein revealed a positive effect of N supplements for all but protein. Additionally, N supplementation increased the fibre degradation of MS, with peptone mainly stimulating hemicellulolytic activity and urea stimulating cellulolytic activity. Moreover, N supplementation tended to increase starch degradation. EMPS of MS was improved by all N supplements, with peptone and urea resulting in the highest increase, followed by AA mix and protein. However, the level of EMPS detected with GS was not achieved. Protozoal 18S rRNA gene copy numbers were negatively correlated with EMPS, whereas no correlation was found between total bacteria and EMPS. Moreover, a stimulating effect of urea, AA mix, and peptone was detected for *Ruminococcus albus* and *Prevotella bryantii*, whereas *Fibrobacter succinogenes* was inhibited by N supplementation. These results indicate that neither the amount of available N nor the N source is the only limiting factor in the low EMPS values of MS *in vitro*. Information is also provided on the stimulating effects of different N sources on several microbial species in mixed rumen culture.

## Introduction

Maize silage (MS) and grass silage (GS) are two of the most important forage sources in total mixed rations fed to highly productive ruminants throughout Europe (1). GS contains more ruminal degradable fibre than MS, and provides substantial amounts of crude protein (CP) that is more rapidly degraded by rumen microbes than CP from MS (1). MS, on the other hand, is characterized by low CP content and high content of readily fermentable carbohydrates; primarily starch. Consequently, the availability of energy and CP for rumen microbes differs between these two types of silage, affecting microbial protein synthesis (MPS) in the rumen (1). A maximum MPS can be achieved by synchronization of the ruminal degradation rate of carbohydrates and proteins, which increases the N usage and efficiency of MPS (EMPS) (2,3). *In vivo* data summarized by Givens and Rulquin (1) suggest a greater EMPS for MS- as compared to GS-based rations. However, *in vitro* data obtained using several total mixed rations and a semi-continuous rumen simulation technique (RUSITEC) showed a negative effect of MS on EMPS (4). These findings were confirmed by Hildebrand et al. in RUSITEC experiments using MS and GS alone or both silages supplemented by 10% (dry matter basis) soybean meal (5,6). Because of the lack of rumino-hepatic N-circulation *in vitro*, the authors hypothesized a deficit in available N as the reason for the lower EMPS in incubating MS as compared to GS (5,6). Indeed, adjustment of the CP content of both silages by supplementation of MS with urea increased the EMPS for MS *in vitro*; however, the higher value detected for GS was not achieved (6). This might indicate a deficit in adequate N sources for microbial growth during *in vitro* incubation of MS. The stimulating effect of amino N (free amino acids (AA), peptides, or protein) on the growth of ruminal bacteria has long been recognized (7), particularly in diets containing high proportions of rapidly fermentable carbohydrates (8-10). However, the fibre-degrading microbial consortium seems to also be stimulated by pre-formed AA, although this effect is mainly attributed to a benefit for synergistic non-cellulolytic partners (11,12). Improved growth of ruminal microbes with supplementation of pre-formed AA in addition to ammonia has been observed under conditions of carbohydrate excess (13). This can be explained by a decrease in energy spilling

(13,14) and reserve carbohydrate synthesis (14), and may be linked to differing requirements of ruminal bacteria for certain AA (15) as well as to positive effects of pre-formed AA on ammonia assimilation (16).

During *in vitro* incubation of MS alone, carbohydrate excess seems probable, in particular when buffer solutions of low ammonia concentrations are used. Thus, we hypothesized that supplementation of MS with an AA mix, peptides, or protein would allow for a higher increase in EMPS than has been reported for urea supplementation (5,6), resulting in a value similar to that of GS. Therefore, the first objective of the present study was to determine whether different amino N sources increase EMPS *in vitro*.

Most of our knowledge about the response of rumen microbes to different N sources is based on pure cultures (15,17-19), which do not reflect the complex interactions of microbial species within a mixed community. Studies on the response of single ruminal species to different N sources in mixed cultures are rare and are restricted to the liquid-associated microbes (16,20), although differences in the use of varying N sources by microbes in the liquid and solid compartments are well known (21). Thus, we also aimed to investigate the response of microbial populations in both rumen compartments to supplementation of MS with different N sources by quantification of total bacteria, methanogens, and protozoa as well as cellulolytic, hemicellulolytic, and proteolytic bacterial species in mixed rumen cultures.

## **Material and Methods**

### **Ethics statement**

The rumen-cannulated cows used as donor animals of inoculum for *in vitro* incubation were housed at the Agricultural Experiment Station of Hohenheim University at Meiereihof in Stuttgart-Hohenheim (Germany), in strict accordance with the German Animal Welfare legislation. All procedures regarding animal handling were approved by the Ethical Commission of Animal Welfare of the Regierungspräsidium Stuttgart, Germany (approval no. A401/14/TE). No animals were sacrificed for this study.

### ***In vitro* experiment and sampling**

The *in vitro* experiments were carried out in a semi-continuous RUSITEC following a previously described procedure (22). Two lactating cows (Jersey) fitted with permanent cannulas were used as donor animals for the inoculum. The cows were fed a total mixed ration containing MS and GS *ad libitum*. Liquid and solid rumen contents were obtained before provision of new feed in the morning. Rumen solids of the two cows were mixed, as were rumen liquids, the latter being additionally filtered through two layers of linen cloth before being used for inoculation. Two RUSITEC systems, each equipped with six fermenter vessels in a water bath (39°C), were used. GS and MS with nutrient specifications as shown in Table 1 served as substrates for incubation. MS either remained non-supplemented or was supplemented with urea ( $\geq 99.5\%$  p.a., Carl Roth, Karlsruhe, Germany), pea peptone ( $>94\%$  CP in dry matter; Pea Peptone A 482; Organotechnie® S.A.S., La Courneuve, France), pea protein isolate ( $>87\%$  CP in dry matter; Emvital® E 7; Emsland Group, Emlichheim, Germany), or a mix of AA ( $>85\%$  CP in dry matter, mixed in accordance with the AA profile of the pea peptone and pea protein) to adjust the N content to that of GS. Pea protein was chosen because of its high ruminal digestibility. Moreover, the pea protein and pea peptone were the purest products available.

**Table 1. Chemical composition of maize silage (MS) and grass silage (GS) used in the study**

	MS	GS
Dry matter (DM), %	94.1	91.3
Nutrients (g kg <sup>-1</sup> DM)		
Crude ash	38	71
Crude protein	69	136
Pure protein	39	79
Neutral detergent fibre <sup>1</sup>	387	477
Acid detergent fibre <sup>2</sup>	219	299
Acid detergent lignin	17	31
Starch	342	-

<sup>1</sup>without residual ash, as determined following  $\alpha$ -amylase pre-treatment

<sup>2</sup>without residual ash

Prior to incubation, the silages were oven-dried (48 h at 40°C) and ground through a 1 mm sieve; 15 g of ground silages were put into individual nylon bags (pore size = 100 µm, Fa. Linker Industrie-Technik GmbH, Kassel, Germany); and urea, AA mix, peptone, or protein were added to the MS. For each treatment, one fermenter per RUSITEC run was used. Five experimental replicates (n = 5) were performed during five experimental runs, for which incubation started on five consecutive weeks. Each RUSITEC run lasted for 13 d. At the beginning of each experimental run, the fermenters with a vessel volume of 800 ml were filled with a 1:1 mixture of rumen liquid and artificial saliva (23) containing 0.7 mmol L<sup>-1</sup> NH<sub>4</sub><sup>+</sup> from NH<sub>4</sub>Cl (enriched with 10.39% <sup>15</sup>N; Campro Scientific GmbH, Berlin, Germany) for calculation of MPS. During the first 24 h of incubation, only one nylon bag filled with feed was added to each fermenter, whereas a second contained pooled rumen solids (60 ± 5 g). The latter was replaced by another feed bag 24 h later. During subsequent days, feedbags were changed at 24-h intervals, so that each feed bag was incubated for 48 h. The artificial saliva used as a buffer was infused continuously at an average (SD) flow rate of 618 (73.4) mL per day per fermenter. Feed containers inside the fermenters were vertically moved by an electric motor at 10 to 12 strokes min<sup>-1</sup>. The effluents of the fermenters were collected in 1-L bottles standing inside an ice-cold water bath. Gas was collected in 10-L bags (Linde PLASTIGAS®-bags, Linde AG, Pullach, Germany) to quantify total gas and methane production. The total gas production was measured as the volume contained in the bags using a drum-type gas meter (TG3; Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Bochum, Germany). Methane concentrations were measured using an infrared methane analyzer (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) as described previously (24).

Sampling (d 7–12) and processing of feed residues from the nylon bags for calculation of degradation of organic matter (OM), crude nutrients, and fibre fractions were as described in detail by Lengowski et al. (25). To determine the MPS, 30 ml of fermenter liquids were collected daily from each fermenter and pooled over days 7 to 13 to obtain liquid-associated microbes by differential centrifugation according to Band and Rohr (26) with modifications described by Wischer et al. (27). Solid-associated microbes were sampled on day 13 by separating from feed residues via methylcellulose according to Boguhn et al. (28). Obtained pellets of liquid- and solid-

associated microbes were frozen at  $-20^{\circ}\text{C}$  until analysis for  $^{15}\text{N}$  enrichment. Total amounts of effluent, gas production, and methane were quantified from d 7 to 13 on a daily basis. Short chain fatty acids (SCFA), ammonia-N, and  $^{15}\text{N}$  enrichment were analyzed in the particle-free fraction of a pooled sample of fermenter effluents (70 mL per d) and treated as described previously (25).

Samples for quantification of microbial populations in fermenter liquids were obtained from d 12 to 13 of incubation. Ten milliliters of fermenter liquids were taken at 2, 4, 12, and 24 h after changing the feed bag on d 12, and 1-mL aliquots were immediately stored at  $-80^{\circ}\text{C}$  until DNA extraction. Additionally, a 30-mL fraction of fermenter liquids taken after 0, 2, 4, 12, and 24 h of incubation was stored at  $-20^{\circ}\text{C}$  for determination of the ammonium concentration. Samples for quantification of microbial populations in feed residues were taken on d 13 at the end of each RUSITEC run from the two feed bags being incubated for 24 and 48 h. Thus, 500 mg of feed residues from each bag were collected and stored at  $-80^{\circ}\text{C}$  until further analysis.

### **Chemical analyses**

Dried (24 h at  $65^{\circ}\text{C}$ ) and pooled (d 7–12) feed residues from the bags were ground to pass through a sieve of 0.5 mm pore size to analyze the concentrations of dry matter, crude ash, (method 3.1), crude protein (method 4.1.1), pure protein (method 4.4.1–4.4.2), neutral detergent fibre (assayed with a thermally stable amylase, method 6.5.1), acid detergent fibre (method 6.5.2), and starch (for MS only, using a polarimetric approach: method 7.2.1) according to the official methods used in Germany (29). Samples of particle-free effluent and fermenter liquid were analyzed for ammonia concentration by steam distillation followed by end-point titration. Concentrations of SCFA in the particle-free effluent were measured by gas chromatography as previously described by Hildebrand et al. (30). Samples of silages, feed residues,  $^{15}\text{NH}_4\text{Cl}$ , freeze-dried microbial pellets, and particle-free effluent were finely ground and analyzed for  $^{15}\text{N}$  and N (microbial pellets only) using an elemental analyzer (EA 1108; Carlo Erba Instruments, Biberach, Germany) combined with an isotope mass spectrometer (MS Finnigan MAT; Thermoquest Italia S.p.A., Milan, Italy). The microbial protein (microbial N multiplied by 6.25) from liquid and solid-associated microbes was calculated as described previously (25). Calculations of nutrient degradation and EMPS were performed as described in detail elsewhere (22).

## Quantification of microbial populations

Quantification of different microbial groups and species in fermenter liquids and feed residues was carried out using real time quantitative PCR (qPCR). Liquid samples of each fermenter taken at different incubation times were thawed and pooled immediately before DNA extraction using 225 µl of each aliquot. Samples of feed residues taken after 24 and 48 h of incubation were also pooled for each fermenter (125 mg of each aliquot). Thus, in total five samples of fermenter liquids and five samples of feed residues per treatment (n=5) were used for analysis of microbial populations. Genomic DNA of microorganisms was extracted using the repeated bead-beating method described by Yu and Morrison (31), with modifications of the protocol as described elsewhere (25). The quality and purity of the DNA extracts were determined spectrophotometrically (NanoDrop UV-Vis spectrophotometer 2000c, Thermo Fisher Scientific), and checked by agarose gel electrophoresis. DNA concentrations were measured fluorometrically using the Qubit 2.0 fluorometer and Qubit dsDNA BR Assay Kit (Life Technologies, Darmstadt, Germany).

For absolute quantification of gene copy numbers, sample-derived DNA standards were generated by conventional PCR. A composite DNA sample was prepared by pooling equal amounts of all DNA extracts obtained from the experiment. The primer pairs and target genes used for quantification of different microbial groups and species are listed in Table 2.

**Table 2. Primer pairs, primer concentration, and annealing temperature (T<sub>a</sub>) used for real time qPCR**

Target organism	Primer	Target gene	T <sub>a</sub> (°C)	Primer conc. (nM)	Reference
Protozoa	316f/539r	18S rRNA	55	900	(32)
Total methanogens	qmcrA-F/qmcrA-R	mcrA <sup>1</sup>	60	900	(33)
Rumen cluster C	mtt For/mtt Rev	mtt <sup>2</sup>	60	550	(34)
Bacteria	338F/805R	16S rRNA	50	300	(35)
<i>Fibrobacter succinogenes</i>	FibSuc3F/FibSuc3R	16S rRNA	58	300	(35)
<i>Ruminococcus albus</i>	Ral1281f/Ral1439r	16S rRNA	55	300	(36)
<i>Prevotella bryantii</i>	PreBry2F/PreBry2R	16S rRNA	61	200	(35)
<i>Selenomonas ruminantium</i>	SelRum2F/SelRum2R	16S rRNA	59	500	(35)
<i>Clostridium aminophilum</i>	S-S-Lac-0435-a-16F/ <i>C. aminophilum</i> R	16S rRNA	56	300	(37,38)

<sup>1</sup>methyl coenzyme M reductase A

<sup>2</sup>TMA-methyltransferase gene of rumen cluster C



PCR was carried out on an iQ<sup>TM</sup>5 Multicolor Real-Time PCR Detection System (Bio-Rad, München, Germany). Reactions were performed in a total volume of 25 µl containing 5 µl of 5 × PCR Mastermix (Bio&SELL, Feucht, Germany), 300–900 nM of each primer (Biomers, Ulm, Germany), 14 ng of template DNA, and nuclease-free water (Qiagen). The amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C (10–30 s), annealing at 50–61°C (15–60 s), elongation at 72°C (30–120 s), and a final extension step at 72°C for 5 min. PCR products were checked for correct fragment length by agarose gel electrophoresis, purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and quantified fluorometrically as described for the DNA extracts. Gene copy numbers of the PCR products were calculated according to Lee et al. (39), and a tenfold serial dilution series of PCR products with 5–6 degrees of dilution was used as standard curves for absolute quantification.

Real time qPCR for quantification of gene copy numbers in the DNA extracts was also carried out on the iQ<sup>TM</sup>5 Multicolor Real-Time PCR Detection System. PCR assays were optimized for MgCl<sub>2</sub> and primer concentrations as well as for annealing temperature. PCR was performed on Framestar 96 well PCR-plates (Bio & Sell) in a total volume of 20 µl. Reaction mixtures contained 4 µl of 5 × *my*-Budget EvaGreenQPCR Mix II (Bio & Sell), 2.5 mM MgCl<sub>2</sub>, 14 ng of template DNA, and primer concentrations as shown in Table 2. The amplification conditions were as follows: initial denaturation at 95°C for 15 min, followed by 35–45 cycles of denaturation at 95°C (10–35 s), annealing at temperatures given in Table 2 (15–60 s), elongation at 72°C (10–120 s), and a final extension step at 72°C for 5 min. Samples were run in duplicate and standards in triplicate. Standard curves were run along with all samples, and for each of the microbial fractions, one plate was used. To check the efficiency of PCR amplification in the samples, a fivefold serial dilution series of the pooled DNA sample of all extracts was run for each primer pair before quantification. PCR efficiencies as determined by the dilution method ranged from 95–108% for the different primer pairs. The specificity of amplification was checked by melting curve analysis. The cycle of quantification (C<sub>q</sub>) was determined using the LinRegPCR quantitative PCR data analysis program (Version 2013.0) (40) on the basis of background-

subtracted fluorescence data generated by real time qPCR. Differences in  $C_q > 0.5$  between the two repeated measures of a sample led to exclusion of the sample from further data analysis. Absolute gene copy numbers in the samples were calculated using the respective standard curves.

### **Statistical analysis**

Statistical analysis was performed using the SAS software package (v. 9.3). Data from different microbial populations were analyzed in two steps. First, gene copy numbers were calculated from  $C_q$  values obtained from LinReg via a plate-specific linear regression estimated using samples from the standard curve. Second, these copy numbers were analyzed using a mixed model approach considering the ‘substrate’ being incubated (S) as the treatment factor as well as block effects from the experimental design of the RUSITEC and laboratory phase. According to the syntax of Patterson (41), the model for the second step can be given as:

$$S + WB + P : R \cdot WB + F + B \cdot P . \quad (1)$$

where WB, R and F denote the block factors ‘water bath’, ‘RUSITEC run’, and ‘fermenter’, respectively. P and B denote ‘plate’ and ‘block within plate’, and are block factors of the laboratory phase. Note that standards were included in the analysis to increase the precision of the P and B effects, but were blocked out from the estimation of effects from the RUSITEC phase via a dummy variable. To simplify the presentation, we dropped this dummy variable in model equations (1) and (3). Fixed effects are presented before the colon, and random effects are listed after the colon. Interactions are denoted by a dot between the corresponding main effects. ‘Water bath’ and ‘plate’ are assumed to be random, but were considered as fixed effects because of the low number of levels. To adjust the varying sample weights used for DNA extraction from feed residues, the sample weight was used as a covariable for the trait ‘gene copy numbers of microbial species in feed residues’. To ensure normal distribution and variance homogeneity of the data, gene copy numbers of *R. albus* in feed residues were subjected to square-root transformation, while the remaining gene copy numbers were log transformed. Least square means from analysis were back-transformed for presentation only. Standard errors were back-transformed using the delta method.

Data on fermentation characteristics were analyzed by fitting a model similar to model 1, but dropping effects from the laboratory phase as follows:

$$S + WB : R \cdot WB + F \quad (2)$$

Data on ammonia concentration measured in fermenter liquids over time (T) were log transformed prior to statistical analysis to reach normal distribution and variance homogeneity. For testing the fixed treatment factors ‘substrate’ and ‘sampling time’, an extended version of model 2 was fitted as follows:

$$S + T + S \cdot T + WB : R \cdot WB + F + F \cdot R + T \cdot WB \cdot R . \quad (3)$$

Because repeated measurements were taken from the same water bath and the same fermenter within a RUSITEC run, we accounted for temporal correlations between observations from the same water bath or fermenter by either fitting a constant covariance or fitting an autoregressive model for these effects if the latter increased the model fit. Model fit was measured via AIC (42). In case of a significant F-test, a multiple *t*-test was used to compare treatments. The level of significance was set at  $\alpha = 0.05$

## Results

### Effect of N source supplemented to MS on fermentation characteristics and EMPS

The pH value measured daily in the fermenter liquids before changing the feed bag ranged from 6.80 to 6.89, and was significantly lower for MS, AA mix, and protein as compared to urea and peptone (Table 3).

**Table 3.** Total gas and methane production, pH, degradation of nutrients after 48 h of incubation, ammonia-N and short-chain fatty acids (SCFA) in the effluent, and efficiency of microbial protein synthesis (EMPS; LS means, *SE*;  $n \geq 4$ )

	MS <sup>1</sup>	MS+ Urea	MS+ AA <sup>2</sup>	MS+ Peptone	MS+ Protein	GS <sup>3</sup>	<i>p</i> -value <sup>4</sup>
<b>pH</b>	6.83 <sup>b</sup> <i>0.04</i>	6.88 <sup>a</sup> <i>0.04</i>	6.82 <sup>b</sup> <i>0.04</i>	6.86 <sup>a</sup> <i>0.04</i>	6.80 <sup>b</sup> <i>0.04</i>	6.89 <sup>a</sup> <i>0.04</i>	0.0001
<b>Total gas, mL d<sup>-1</sup></b>	819 <sup>ab</sup> <i>84</i>	790 <sup>bc</sup> <i>83</i>	957 <sup>a</sup> <i>86</i>	938 <sup>ab</sup> <i>80</i>	843 <sup>ab</sup> <i>79</i>	640 <sup>c</sup> <i>80</i>	0.0046
<b>Methane, %</b>	11.5 <sup>b</sup> <i>0.8</i>	12.9 <sup>b</sup> <i>0.8</i>	11.5 <sup>b</sup> <i>0.8</i>	12.5 <sup>b</sup> <i>0.8</i>	11.5 <sup>b</sup> <i>0.8</i>	16.0 <sup>a</sup> <i>0.8</i>	0.0003
<b>Methane, mL d<sup>-1</sup></b>	95 <i>13</i>	106 <i>13</i>	105 <i>13</i>	122 <i>13</i>	100 <i>13</i>	108 <i>13</i>	0.3434
<b>Methane, mL g<sup>-1</sup> fermented OM<sub>corr</sub><sup>5</sup></b>	17 <i>3</i>	18 <i>3</i>	19 <i>3</i>	22 <i>2</i>	21 <i>3</i>	20 <i>2</i>	0.3826
<b>Degradation, %</b>							
OM <sub>corr</sub> <sup>5</sup>	40 <sup>c</sup> <i>0.5</i>	45 <sup>a</sup> <i>0.5</i>	45 <sup>a</sup> <i>0.5</i>	44 <sup>a</sup> <i>0.5</i>	40 <sup>c</sup> <i>0.5</i>	42 <sup>b</sup> <i>0.5</i>	<0.0001
CP <sub>corr</sub> <sup>5</sup>	59 <sup>d</sup> <i>1.2</i>	76 <sup>a</sup> <i>1.2</i>	66 <sup>c</sup> <i>1.2</i>	69 <sup>b</sup> <i>1.1</i>	38 <sup>f</sup> <i>1.1</i>	55 <sup>c</sup> <i>1.1</i>	<0.0001
NDF <sup>6</sup>	10.5 <sup>c</sup> <i>0.7</i>	11.0 <sup>c</sup> <i>0.7</i>	11.4 <sup>bc</sup> <i>0.7</i>	13.0 <sup>b</sup> <i>0.7</i>	10.9 <sup>c</sup> <i>0.7</i>	18.9 <sup>a</sup> <i>0.7</i>	<0.0001
ADF <sup>7</sup>	5 <sup>d</sup> <i>0.5</i>	9 <sup>b</sup> <i>0.5</i>	6 <sup>cd</sup> <i>0.5</i>	7 <sup>c</sup> <i>0.5</i>	7 <sup>c</sup> <i>0.5</i>	19 <sup>a</sup> <i>0.5</i>	<0.0001
Starch	64 <i>1.5</i>	70 <i>1.5</i>	68 <i>1.5</i>	68 <i>1.4</i>	67 <i>1.5</i>	. <i>.</i>	0.0648
<b>SCFA, mmol d<sup>-1</sup></b>							
Total	32.1 <sup>b</sup> <i>0.88</i>	34.4 <sup>a</sup> <i>0.88</i>	35.2 <sup>a</sup> <i>0.88</i>	33.5 <sup>ab</sup> <i>0.88</i>	33.5 <sup>ab</sup> <i>0.88</i>	27.9 <sup>c</sup> <i>0.88</i>	<0.0001
Acetate	13.3 <sup>b</sup> <i>0.48</i>	14.7 <sup>a</sup> <i>0.48</i>	14.4 <sup>ab</sup> <i>0.48</i>	13.2 <sup>b</sup> <i>0.45</i>	14.2 <sup>ab</sup> <i>0.45</i>	11.7 <sup>c</sup> <i>0.45</i>	0.0023
Propionate	7.01 <i>0.37</i>	7.14 <i>0.37</i>	7.00 <i>0.37</i>	7.05 <i>0.37</i>	6.97 <i>0.37</i>	7.18 <i>0.37</i>	0.9835
Isobutyrate	0.25 <sup>d</sup> <i>0.01</i>	0.24 <sup>d</sup> <i>0.01</i>	0.45 <sup>a</sup> <i>0.01</i>	0.41 <sup>b</sup> <i>0.01</i>	0.27 <sup>d</sup> <i>0.01</i>	0.37 <sup>c</sup> <i>0.01</i>	<0.0001
Butyrate	7.27 <sup>c</sup> <i>0.21</i>	8.11 <sup>ab</sup> <i>0.21</i>	8.20 <sup>a</sup> <i>0.21</i>	7.90 <sup>ab</sup> <i>0.21</i>	7.68 <sup>bc</sup> <i>0.21</i>	4.61 <sup>d</sup> <i>0.21</i>	<0.0001
Isovalerate	1.48 <sup>bc</sup> <i>0.19</i>	1.50 <sup>bc</sup> <i>0.19</i>	1.93 <sup>a</sup> <i>0.20</i>	1.68 <sup>ab</sup> <i>0.19</i>	1.37 <sup>c</sup> <i>0.19</i>	1.28 <sup>c</sup> <i>0.19</i>	0.0011
Valerate	2.74 <sup>b</sup> <i>0.16</i>	2.79 <sup>b</sup> <i>0.16</i>	3.20 <sup>a</sup> <i>0.16</i>	3.21 <sup>a</sup> <i>0.16</i>	2.95 <sup>ab</sup> <i>0.16</i>	2.76 <sup>b</sup> <i>0.16</i>	0.0054
Acetate:propionate	1.93 <sup>a</sup> <i>0.11</i>	2.06 <sup>a</sup> <i>0.11</i>	2.07 <sup>a</sup> <i>0.11</i>	1.89 <sup>a</sup> <i>0.11</i>	2.06 <sup>a</sup> <i>0.11</i>	1.64 <sup>b</sup> <i>0.11</i>	0.0064
<b>NH<sub>3</sub>-N, mmol d<sup>-1</sup></b>	2.1 <sup>d</sup> <i>0.17</i>	8.2 <sup>a</sup> <i>0.17</i>	6.1 <sup>b</sup> <i>0.17</i>	5.9 <sup>b</sup> <i>0.17</i>	2.5 <sup>d</sup> <i>0.17</i>	3.4 <sup>c</sup> <i>0.17</i>	<0.0001
<b>MPS<sup>8</sup>, mg d<sup>-1</sup></b>	609 <sup>d</sup> <i>15.8</i>	1077 <sup>a</sup> <i>15.8</i>	1010 <sup>b</sup> <i>15.8</i>	1091 <sup>a</sup> <i>15.8</i>	708 <sup>c</sup> <i>15.8</i>	1065 <sup>a</sup> <i>15.8</i>	<0.0001
<b>EMPS<sup>9</sup></b>	111 <sup>c</sup> <i>2.4</i>	174 <sup>b</sup> <i>2.4</i>	155 <sup>c</sup> <i>2.4</i>	171 <sup>b</sup> <i>2.4</i>	123 <sup>d</sup> <i>2.4</i>	200 <sup>a</sup> <i>2.4</i>	<0.0001
<b>Efficiency of N utilization<sup>10</sup>, %</b>	100 <sup>a</sup> <i>2.00</i>	76 <sup>d</sup> <i>2.01</i>	81 <sup>c</sup> <i>2.00</i>	86 <sup>b</sup> <i>1.97</i>	98 <sup>a</sup> <i>1.97</i>	101 <sup>a</sup> <i>1.97</i>	<0.0001

<sup>1</sup>Maize silage

<sup>2</sup>Amino acid mix

<sup>3</sup>Grass silage

<sup>4</sup>The ANOVA *p*-value shown is the *p*-value for an F-test of substrate differences.

<sup>5</sup>Organic matter and crude protein corrected for contribution of microbes attached to feed residues

<sup>6</sup>Neutral detergent fibre without residual ash determined after  $\alpha$ -amylase pre-treatment

<sup>7</sup>Acid detergent fibre

<sup>8</sup>Microbial protein synthesis, sum of microbial CP from fermenter liquids, and feed residues

<sup>9</sup>Gram microbial CP kg<sup>-1</sup> fermented OM

<sup>10</sup>(Grams of microbial N/grams of available N) \* 100

<sup>a-f</sup>Different lowercase letters indicate significant differences between treatments.

Total gas production was lowest when MS was supplemented with urea and highest when it was supplemented with AA mix, whereas peptone, protein, and MS alone results were intermediate. The methane proportion in total gas was on average (SE) 12 (0.8)% for MS treatments being not affected by the N source. Methane production was, on average, 106 mL d<sup>-1</sup> (13.0) or 19.5 (2.5) mL g<sup>-1</sup> of fermented OM, with no significant difference between treatments ( $P > 0.05$ ).

The degradation of OM was highest for supplementation of MS with urea, AA mix, and peptone, at 45, 45, and 44%, respectively (Table 3). In the cases of MS alone or MS supplemented with protein, around 5% less OM was degraded. The degradation of CP ranged from 38 to 76% and was highest for urea supplementation; followed by peptone, AA mix, MS alone, and protein.

The degradation of NDF was lowest for MS alone, and a significant increase from 10.5 to 13% was observed with peptone supplementation. Degradation of ADF was also lowest for MS alone, but supplementing peptone, protein, or urea increased ADF degradation to 7 and 9%, respectively, whereas no effect of AA mix was observed. The degradation of starch from MS was on average 67 (1.5)%, and was not significantly affected by N supplementation or N source.

The total amount of SCFAs produced was increased by supplementation of AA mix or urea to MS, whereas no effect was found for peptone and protein. N supplementation did not affect propionate production, but acetate production was increased by urea. Isobutyrate production was highest for supplementation of MS with AA mix, followed by peptone, protein, urea, and MS alone, with no statistical differences found among the latter three. Among MS treatments,

production of butyrate was lowest for MS alone and protein, whereas urea, AA mix, and peptone supplementation resulted in a significantly higher production of butyrate as compared to MS alone. Production of isovalerate and valerate was similar for MS alone and MS supplemented with urea or protein. However, supplementation of AA mix and peptone increased the production of isovalerate and valerate in comparison to MS alone.

The daily production of ammonia-N when MS was incubated alone was on average 2.1 mmol (Table 3). A similar value was found for protein supplementation, but AA mix, peptone, and urea significantly increased ammonia-N production to 6.1, 5.9, and 8.2 mmol d<sup>-1</sup>, respectively. The ammonia concentration in the fermenter liquid during the last 24 h of incubation was highest for urea supplementation at all sampling times, followed by peptone and AA mix; the latter differed only from urea at sampling time '12 h after change of feed bag' (Fig 1). MS alone and protein supplementation resulted in the lowest ammonia concentrations in the fermenter liquids. Differences between these two treatments were only observed at sampling time '4 h after change of feed bag', with a lower concentration being measured for MS alone as compared to protein. For all treatments except urea up to '12 h after change of feed bag', the ammonia concentration in the fermenter liquid decreased, followed by an increase during the next 12 hours (for protein only numerically). However, in none of the treatments the initial ammonia concentration was achieved '24 h after change of feed bag'. In the case of supplementation with urea, the ammonia concentration decreased only within the first two hours after changing the feed bag and remained at this level during subsequent incubation.

**Fig 1. Ammonia concentration in fermenter liquids measured at different sampling times after adding a new feed bag**

MS (maize silage, yellow), MS+Urea (black), MS+AA mix (red), MS+Peptone (blue), MS+Protein (orange), GS (grass silage, green; LS means, SD, back-transformed, n ≥ 4).

MPS was affected by N supplementation as well as by N source. For MS alone, MPS was on average 609 mg d<sup>-1</sup>; the lowest value among the treatments. With protein supplementation, MPS was 99 mg d<sup>-1</sup> higher than for MS alone, and urea, AA mix, and peptone led to a further increase,

resulting in MPS of 1077, 1010, and 1091 mg d<sup>-1</sup>, respectively. Based on these results, the lowest EMPS among MS treatments was calculated for MS alone and the highest for urea and peptone. By contrast, the efficiency of N utilization was lowest for urea and highest for MS alone, protein, and GS.

### **Effect of N source on microbial populations**

Numbers of all microbial populations investigated were significantly affected by the N source supplemented to MS. The 18S rRNA gene copy number of protozoa in the fermenter liquid and feed residues was highest when MS alone was incubated, whereas protein supplementation reduced protozoal gene copy numbers by half in fermenter liquids (Fig 2). A further decrease in both microbial fractions was observed for peptone, urea, and AA mix.

### **Fig 2. 18S rRNA, 16S rRNA, and mcrA gene copy numbers of protozoa, bacteria, methanogens, and Archaea belonging to rumen cluster C (RCC) detected in fermenter liquid (white) and feed residues (grey)**

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed,  $n \geq 4$ ).

At the domain level of bacteria, protein increased total numbers in fermenter liquids by around 50% but decreased counts by one-third in feed residues compared to MS alone (Fig 2). Moreover, supplementing AA mix to MS increased numbers of bacteria in the fermenter liquids compared to supplementation of urea.

Total numbers of methanogens in fermenter liquids were only affected by supplementation of peptone, which led to lower numbers compared to the other MS treatments (Fig 2). In feed residues total methanogens were increased by supplementation of urea, peptone and protein compared to MS alone while the AA treatment was intermediate. Similar observations were made for methanogens affiliated with rumen cluster C (RCC), although supplementation of urea resulted in lower numbers than MS alone and protein (Fig 2).

Compared to MS alone, N supplementation did not change the number of *F. succinogenes* in the fermenter liquids (Fig 3), but protein supplementation resulted in significantly higher numbers than urea, AA mix, or peptone supplementation. In feed residues, numbers of *F. succinogenes* were highest for MS alone and AA mix, and supplementation of peptone, urea, and protein led to a decrease of 40–60% in *F. succinogenes* counts compared to MS alone.

**Fig 3. 16S rRNA gene copy numbers of *Fibrobacter succinogenes* and *Ruminococcus albus* in fermenter liquid (white) and feed residues (grey)**

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed,  $n \geq 3$ ).

The abundance of *R. albus* in the fermenter liquids was increased by all four N sources, and urea and peptone led to even higher numbers than protein did (Fig 3). In feed residues, supplementation of MS with AA mix or peptone resulted in about three-fold higher numbers of *R. albus* than MS alone, whereas the remaining MS treatments did not differ from each other.

In fermenter liquids, *P. bryantii* numbers were lowest for MS alone and AA mix (Fig 4). Neither peptone nor protein statistically differed from MS alone. However, supplementation of urea resulted in two-fold higher numbers of *P. bryantii* in fermenter liquids as compared to MS. Similar observations were made in feed residues.

**Fig 4. 16S rRNA gene copy numbers of *Prevotella bryantii*, *Selenomonas ruminantium*, and *Clostridium aminophilum* detected in fermenter liquid (white) and feed residues (grey)**

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed,  $n \geq 4$ ).

No effect of the N source supplemented to MS was detected for numbers of *S. ruminantium* in fermenter liquids (Fig 4). In contrast, protein significantly decreased the number of *S. ruminantium* in feed residues by around one-third compared with MS alone, whereas urea, AA mix, and peptone treatments did not differ from MS alone or from protein.



In comparison to MS alone, supplementation of peptone increased the number of *C. aminophilum* in fermenter liquids by around 50%, whereas urea and AA mix lowered its numbers by 60 and 40%, respectively (Fig 4). No effect of N supplementation or N source on the abundance of *C. aminophilum* in feed residues was found.

### **Comparison of MS treatments with GS**

In the case of GS incubation, degradation of OM and CP and production of isobutyrate and ammonia were intermediate compared to the MS treatments (Table 3). Production of total gas, total SFCA, acetate, and butyrate was lower for GS than for MS treatments, whereas methane concentration, degradation of fibre fractions, and EMPS were higher. Moreover, incubation of GS resulted in a higher pH than measured for MS alone and MS supplemented with AA or protein. Isovalerate and valerate production was only decreased for GS compared to MS supplemented with AA mix or peptone. The amount of propionate and methane produced per day was similar for all treatments, irrespective of silage. Incubation of GS decreased the gene copy numbers of protozoa, total bacteria (Fig 2), *S. ruminantium*, and *C. aminophilus* (Fig 4) compared to the MS treatments, whereas methanogens (Fig 2) and *P. bryantii* (Fig 4) seemed to be favored by GS.

## **Discussion**

### **Impact of N source on nutrient degradation and consequences for EMPS of MS**

The differences we found in nutrient degradation and EMPS between GS and non-supplemented MS are in accordance with the results of former *in vitro* studies (6,25). Our results also confirm the previous observation that supplementation of MS with urea leads to an increase in degradation of OM and MPS (6) and to smaller differences in EMPS between MS and GS (6,28). The higher degradation of OM with urea supplementation can largely be explained by an increase in the degradation of CP, which is attributed to the high availability of urea-N for rumen microbes. The breakdown of ADF was also enhanced by urea, and the degradation of starch tended to be higher (Table 3). This indicates an inadequate N supply for cellulolytic and amylolytic species when MS was incubated alone. These observations are in accordance with the results of previous studies in continuous cultures (43,44), but contrast with recent *in vitro* studies by Hildebrand et al. (6), in which the positive effect of urea supplementation to MS was limited to starch degradation.

Adding peptone to MS resulted in an EMPS similar to that found for urea, although the efficiency of N utilization was higher for peptone. This confirms the results of Griswold et al. (45), who incubated oat straw and maize kernels with different N sources in a continuous culture system. However, in the present study, ADF degradation was increased less by peptone than by urea, whereas NDF degradation was considerably increased. This contrasts with results of Griswold and colleagues (45), who reported much lower ADF degradation for urea than for peptide supplementation and no differences in NDF degradation. These differences between studies may be a result of the different substrates used. However, the higher degradation of OM with peptone resulted primarily from an increase in the degradation of CP, which was accompanied by increased production of isoacids and higher release of ammonia as compared to MS alone. This indicates a higher fermentation of AA C skeletons (probably from peptone) and is consistent with former studies (9,45). For GS, less intense AA fermentation as indicated by the lower isoacid production and higher efficiency of N utilization were observed than for MS+peptone, thereby, in combination with the moderate degradation of OM, explaining the higher EMPS detected for GS. The release of fibre-bound amino N during fibre degradation probably led to a more synchronized supply of energy and N for microbes from GS than from external N sources supplemented to MS. Supplementation with AA mix resulted in a similar production of isoacids and ammonia to that observed for peptone, although degradation of CP was lower with the AA mix. This indicates less efficient utilization of free AA for MPS than of peptides, confirming the results of previous *in vitro* and *in vivo* studies (9,17,46,47). Moreover, AA mix only minimally increased degradation of starch and fiber, thereby leading to an MPS and EMPS that were higher than that of MS alone but lower than those of peptone and urea.

The low increase in EMPS observed with protein seemed to be related to very low hydrolysis of the supplemented protein, as CP degradation was considerably decreased. Griswold et al. (45) also reported much lower degradation of N when soy protein isolate was added to oat straw and maize kernels instead of urea, AA, or peptides. The authors assumed the rate of protein hydrolysis to be more limiting than the rate of peptide uptake by microorganisms. Normally, pea protein is highly soluble and is rapidly degraded in the rumen (48). However, the process of protein

isolation may have caused structural changes that negatively affected its degradation. Nevertheless, the total amount of CP degraded was higher with MS+protein compared to MS alone thus indicating a higher N availability with supplementation of the protein. This enhanced ADF degradation and thus allowed for a higher MPS and EMPS than found for MS alone.

Regardless of N supplementation the overall level of fibre degradation from MS was noticeably low in the RUSITEC. This has been observed also in former studies (6, 25, 30). It remains to be clarified in future work whether inhibition of fibre degradation in MS incubation was caused by a higher accumulation of fermentation products (6) or a less adaptability of fibre degrading microbes.

### **Impact of N source on the abundance of microbial populations**

Consistent with former *in vitro* studies on MS and GS (25,49), the higher EMPS detected for GS and N-supplemented MS could not be attributed to higher bacterial numbers or to a decrease in the degradation of OM. Calculations of the overall gene copy number of microbes in the fermenters (sum of total bacteria, methanogens, and protozoa in both fractions) from the present study also revealed no correlation with the MPS or EMPS (data not shown). Diurnal changes in the microbial populations of the fermenter liquids were taken into account by repeated sampling, but populations associated with feed residues could only be analyzed after 24 and 48 h of incubation. Thus, possible changes during the first hours after providing new substrate were not considered. Determination of the MPS in both microbial fractions was also based on spot-sampling only (24 h or 24 and 48 h after change of feed bag for liquids and feed residues, respectively). Moreover, substrate-dependent shifts in microbial populations were evident. Thus, differences in the CP content of microbes or rRNA operon copy numbers may have contributed to the inconsistency found between MPS and total microbial numbers.

As confirmed by our results, total numbers of methanogens are not necessarily related to methane production (50). Nonetheless, higher numbers of total methanogens coincided with increased fibre degradation (Fig 5). This is consistent with the overall higher number of hydrogen-producing cellulolytic *R. albus* in the case of GS incubation and N-supplemented MS treatments. *R. albus* prefers ammonia or urea as its main N source (15,51), whereas growth yields on peptides are

lower (52). No growth was observed with AA as the sole N source (51), and the growth-stimulating effects of AA and peptides have been shown on cellobiose but not on cellulose (15). Hence, the increased numbers of *R. albus* with supplementation of AA mix and peptone might be explained by stimulation of non-cellulolytic partners involved in fibre degradation rather than by a direct effect of these N sources on this species (12). Non-cellulolytic partners in the fibre-degrading consortium are important because they remove products formed during cellulose breakdown, thus preventing feedback inhibition of cellulolysis (52). Such a non-cellulolytic species being involved in fibre degradation is *P. bryantii*, the growth of which can be stimulated by peptides and AA (17). This may explain the numerical increase of *P. bryantii* with supplementation of peptides or AA to MS in the present study. However, a significant increase in numbers of *P. bryantii* was observed only with supplementation of urea, probably because its main N source for CP synthesis seems to be ammonia (17).

**Fig 5. Pearson correlation between total numbers of methanogens in feed residues and degradation of neutral ( $r = 0.89$ ,  $P = 0.017$ ) and acid detergent fibre ( $r = 0.81$ ,  $P = 0.048$ )**

The growth of *F. succinogenes* was positively affected by AA and peptides in pure culture (15). However, this could not be confirmed in the mixed environment of the present study. *F. succinogenes* counts decreased with N supplementation, possibly due to competition with *R. albus* for substrate (53). Wang et al. (16) also detected inverse trends in abundance of *F. succinogenes* and *R. albus* with supplementation of ammonia and free AA to batch cultures of mixed rumen microbes from goats fed a maize-wheat-bran-based diet. Calculations of the overall numbers of both cellulolytics in the fermenters showed no correlation to fibre degradation (data not shown). Thus, differences in cellulose degrading activity or the presence of cellulolytics other than *R. albus* contributed to the increased fibre degradation with N supplementation of MS. Moreover, with supplementation of amino N to MS a higher availability of isoacids could have contributed to the increased fibre degradation, as beneficial effects of isoacids on cellulolytics are well known (54). However, the highest ADF degradation among MS treatments was found for urea supplementation, which had a similar level of isoacid production as MS alone (~2.9 mM). Thus, the level of isoacids seemed to be less important for fibre degradation under the given conditions

of *in vitro* incubation. This is consistent with previous studies that show a concentration of 0.30 mM to be adequate for cellulose digestion (54).

Protozoal 18S rRNA gene copy numbers were negatively correlated with EMPS (Fig 6). Defaunation can positively affect EMPS as a result of both lower digestion of OM and higher net synthesis of microbial CP (55,56). The former effect is associated with lower fibre degradation due to the loss of protozoal fibrolytic activity, whereas the latter is mainly related to an increase in bacterial numbers due to lower predation (56). However, these effects were not observed under the *in vitro* conditions of the present study. It has recently been shown that MPS is inefficient, largely owing to maintenance functions, accumulation of reserve carbohydrates, and energy spilling (14). Protozoa show higher energetic requirements for maintenance than bacteria (14,57) and under modest carbohydrate excess, they accumulate more reserve carbohydrates (14). Thus, the presence of protozoa is thought to have a negative impact on the overall energetic efficiency of the rumen ecosystem, especially when low protein diets are fed (56). However, the question remains if this considerably contributed to the EMPS under energy-limited conditions of RUSITEC incubation which is normally associated with overall low numbers of protozoa present in the fermenters. Protozoa engulf bacteria as their main source of protein (57) and ingest insoluble dietary protein (55,58), but they possess limited ability to assimilate ammonia (59), AA, or peptides (58). This may explain the success of protozoa on MS alone and with supplementation of protein.

**Fig 6. Pearson correlation between protozoal 18S rRNA gene copy numbers in fermenter liquid (open;  $r = -0.85$ ,  $P = 0.034$ ) or feed residues (filled;  $r = -0.82$ ,  $P = 0.048$ ) and the efficiency of microbial protein synthesis (EMPS, g microbial crude protein kg<sup>-1</sup> fermented organic matter)**

*C. aminophilum* uses only AA and peptides as energy sources (60), thus explaining the positive effect of peptone on this species. However, AA mix supplemented to MS inhibited the growth of *C. aminophilum* as compared to MS alone (fermenter liquid:  $P = 0.034$ ; feed residues:  $P = 0.006$ ), although in pure culture this bacterium is known to grow more rapidly on AA than on peptides

(60). These contradictory findings may be due to increased interaction and competition with other microbial species in the mixed rumen as compared to pure culture.

*S. ruminantium* uses a wide variety of N sources (61), but its incorporation of free AA is relatively low (8,17); thus, ammonia is assumed to be the main N source of this species (17,61). However, free AA and peptides stimulate the growth of *S. ruminantium* in pure culture (8,17) and decrease its *de novo* synthesis of AA (17). We did not observe a positive effect of AA mix and peptides on *S. ruminantium*, which might be an effect of the mixed rumen culture and incubation of MS, which is more complex in nutrient composition than media used for pure cultures. Therefore, it appears that the AA, peptide, and N supply of MS was sufficient for the growth of *S. ruminantium*. However, an inhibiting effect of protein on *S. ruminantium* in feed residues was observed, likely due to competition for energy sources with other microbial species favored by the addition of protein.

### **Conclusions**

Contrary to our hypothesis, supplementation of MS with amino N did not result in an EMPS higher than that found with urea. None of the N sources increased EMPS to the level achieved with GS. With peptone supplementation, an increase in EMPS similar to that of urea was achieved, but the efficiency of N utilization was higher than with urea. Whereas urea supplementation mainly increased the degradation of cellulose and starch, peptone primarily favored hemicellulolytic activity. Moreover, our data suggest a different response of *F. succinogenes*, *C. aminophilum*, and *S. ruminantium* to AA and peptides in the RUSITEC than formerly shown in pure cultures. This might be associated with different conditions in mixed cultures and the more complex substrate used with MS.

### **Acknowledgments**

The authors would like to thank Dr. H. Steingäß for assistance in planning the experiment and helpful discussions about data analysis.

### **References**

(1) Givens DI, Rulquin H. Utilisation by ruminants of nitrogen compounds in silage-based diets. Anim Feed Sci Tech 2004;114(1-4):1-18.

- (2) Kaswari T, Lebzien P, Flachowsky G, ter Meulen U. Studies on the relationship between the synchronization index and the microbial protein synthesis in the rumen of dairy cows. *Anim Feed Sci Tech* 2007;139(1-2):1-22.
- (3) Cole NA, Todd RW. Opportunities to enhance performance and efficiency through nutrient synchrony in concentrate-fed ruminants. *J Anim Sci* 2008;86(14 Suppl).
- (4) Boguhn J, Kluth H, Rodehutsord M. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes *in vitro*. *J Dairy Sci* 2006;89(5):1592-1603.
- (5) Hildebrand B, Boguhn J, Rodehutsord M. Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. *Animal* 2011;5(4):537-546.
- (6) Hildebrand B, Boguhn J, Rodehutsord M. Investigations on the effect of forage source, grinding, and urea supplementation on ruminal fermentation and microbial protein flow in a semi-continuous rumen simulation system. *Arch Tierernahr* 2011;65(5):402-414.
- (7) Russell JB. The energy spilling reactions of bacteria and other organisms. *J Mol Microbiol Biotechnol* 2007;13(1-3):1-11.
- (8) Cruz Soto RC, Muhammed SA, Newbold CJ, Stewart CS, Wallace RJ. Influence of peptides, amino acids and urea on microbial activity in the rumen of sheep receiving grass hay and on the growth of rumen bacteria *in vitro*. *Anim Feed Sci Tech* 1994;49(1-2):151-161.
- (9) Carro MD, Miller EL. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semicontinuous culture system (RUSITEC). *Brit J Nutr* 1999;82(2):149-157.
- (10) Chikunya S, Newbold CJ, Rode L, Chen XB, Wallace RJ. Influence of dietary rumen-degradable protein on bacterial growth in the rumen of sheep receiving different energy sources. *Anim Feed Sci Tech* 1996;63(1-4):333-340.
- (11) Firkins JL. Reconsidering rumen microbial consortia to enhance feed efficiency and reduce environmental impact of ruminant livestock production systems. *Rev Bras Zootec* 2010;39(Suppl. 1):445-457.
- (12) Newbold CJ. The need for nitrogen. *Brit J Nutr* 1999;82(2):81-82.
- (13) Van Kessel JS, Russell JB. The effect of amino nitrogen on the energetics of ruminal bacteria and its impact on energy spilling. *J Dairy Sci* 1996;79(7):1237-1243.
- (14) Hackmann TJ, Firkins JL. Maximizing efficiency of rumen microbial protein production. *Front Microbiol* 2015;6:465.
- (15) Atasoglu C, James Newbold C, John Wallace R. Incorporation of [15N] ammonia by the cellulolytic ruminal bacteria *Fibrobacter succinogenes* BL2, *Ruminococcus albus* SY3, and *Ruminococcus flavefaciens* 17. *Appl Environ Microb* 2001;67(6):2819-2822.
- (16) Wang P, Tan Z, Guan L, Tang S, Zhou C, Han X, et al. Ammonia and amino acids modulates enzymes associated with ammonia assimilation pathway by ruminal microbiota *in vitro*. *Livest Sci* 2015;178:130-139.

- (17) Atasoglu C, Valdés C, Walker ND, Newbold CJ, Wallace RJ. *De novo* synthesis of amino acids by the ruminal bacteria *Prevotella bryantii* B14, *Selenomonas ruminantium* HD4, and *Streptococcus bovis* ES1. Appl Environ Microb 1998;64(8):2836-2843.
- (18) J.N. Kim, E. DeCrescenzo Henriksen, I.K.O. Cann, R.I. Mackie, Nitrogen Utilization and Metabolism in *Ruminococcus albus* 8, Appl. Environ. Microbiol. 80 (2014) 3095-3102.
- (19) Russell JB. Effect of amino acids on the heat production and growth efficiency of *Streptococcus bovis*: Balance of anabolic and catabolic rates. Appl Environ Microb 1993;59(6):1747-1751.
- (20) Wang WJ, Yang WR, Wang Y, Song EL, Liu XM, Wan FC. Effects of soybean small peptides on rumen fermentation and on intestinal and total tract digestion of Luxi Yellow cattle. Asian-Australas J Anim Sci 2013;26(1):72-81.
- (21) Carro MD, López S, Valdés C, González JS. Effect of nitrogen form (casein and urea) on the *in vitro* degradation of cell walls from six forages. J, Anim Physiol Anim Nutr 1999;81(4-5):212-222.
- (22) Boguhn J, Kluth H, Rodehutsord M. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis *in vitro*. J Dairy Sci 2006;89(5):1580-1591.
- (23) McDougall EI. Studies on ruminant saliva. 1. The composition and output of sheeps saliva. Biochem J 1948;43(1):99-109.
- (24) Wischer G, Boguhn J, Steingaß H, Schollenberger M, Rodehutsord M. Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis *in vitro*. Animal 2013;7(11):1796-1805.
- (25) Lengowski MB, Zuber KHR, Witzig M, Möhring J, Boguhn J, Rodehutsord M. Changes in rumen microbial community composition during adaption to an *in vitro* system and the impact of different forages. PLoS ONE 2016;11(2).
- (26) Brandt M, Rohr K. Beiträge zur Quantifizierung der N-Umsetzung in den Vormägen von Milchkühen 1. Mitteilung: Bestimmung des Mikrobenstickstoffs im Duodenalchymus mit Hilfe von <sup>15</sup>N. Z Tierphysiol Tierernähr Futtermittelkd. 1981; 46:39–48.
- (27) Wischer G, Boguhn J, Steingaß H, Schollenberger M, Hartung K, Rodehutsord M. Effect of monensin on *in vitro* fermentation of silages and microbial protein synthesis. Arch Anim Nutr. 2013; 67(3):219-234. doi: 10.1080/1745039X.2013.793050 PMID: 23679006
- (28) Boguhn J, Zuber T, Rodehutsord M. Effect of donor animals and their diet on *in vitro* nutrient degradation and microbial protein synthesis using grass and corn silages. J Anim Physiol An N 2012;97(3):547-557.
- (29) Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA). Die chemische Untersuchung von Futtermitteln mit 1. - 8. Ergänzungslieferung (1983 - 2012), 3. Auflage, VDLUFA-Verlag, Darmstadt. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch) 1976 (Bd. III).
- (30) Hildebrand B, Boguhn J, Rodehutsord M. Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation *in vitro*. Animal 2011;5(4):528-536.



- (31) Yu ZT, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 2004;36(5):808-812.
- (32) Sylvester JT, Karnati SKR, Yu Z, Morrison M, Firkins JL. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J Nutr* 2004;134(12):3378-3384.
- (33) Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol* 2006;58(3):572-582.
- (34) Poulsen M, Schwab C, Borg Jensen B, Engberg RM, Spang A, Canibe N, et al. Methylophilic methanogenic *Thermoplasmata* implicated in reduced methane emissions from bovine rumen. *Nat Commun* 2013;4.
- (35) Stevenson DM, Weimer PJ. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl Microbiol Biot* 2007;75(1):165-174.
- (36) Koike S, Kobayashi Y. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol Lett* 2001;204(2):361-366.
- (37) Kong Y, He M, McAlister T, Seviour R, Forster R. Quantitative fluorescence *in situ* hybridization of microbial communities in the rumens of cattle fed different diets. *Appl Environ Microb* 2010;76(20):6933-6938.
- (38) Krause DO, Russell JB. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl Environ Microb* 1996;62(3):815-821.
- (39) Lee C, Lee S, Shin SG, Hwang S. Real-time PCR determination of rRNA gene copy number: Absolute and relative quantification assays with *Escherichia coli*. *Appl Microbiol Biot* 2008;78(2):371-376.
- (40) Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB, et al. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* 2009;37(6):e45, doi:10.1093/nar/gkp045.
- (41) Patterson HD. Analysis of series of variety trials. In: RA Kempton, PN Fox, editors, *Statistical methods for plant variety evaluation*, London: Chapman and Hall 1997:139-161.
- (42) Wolfinger R. Laplace's approximation for nonlinear mixed models. *Biometrika* 1993;80(4):791-795.
- (43) Griswold KE, Apgar GA, Bouton J, Firkins JL. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. *J Anim Sci* 2003;81(1):329-336.
- (44) Windschitl PM, Stern MD. Effects of urea supplementation of diets containing lignosulfonate-treated soybean meal on bacterial fermentation in continuous culture of ruminal contents. *J Anim Sci*. 1988;66:2948-2958.

- (45) Griswold KE, Hoover WH, Miller TK, Thayne WV. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. *J Anim Sci* 1996;74(2):483-491.
- (46) Argyle JL, Baldwin RL. Effects of amino acids and peptides on rumen microbial growth yields. *J Dairy Sci* 1989;72(8):2017-2027.
- (47) Brooks MA, Porter JH, Kerley MS. Assessment of amino acid supplementation on rumen microbial efficiency and nitrogen metabolism using a continuous-culture system. *Prof Anim Sci* 2011;27:152-159.
- (48) Andrade-Montemayor H, Garcia-Gasca T, Kawas J. Ruminal fermentation modification of protein and carbohydrate by means of roasted and estimation of microbial protein synthesis. *Rev Bras Zootec* 2009;38(Suppl. 1):277-291.
- (49) Witzig M, Boguhn J, Zeder M, Seifert J, Rodehutsord M. Effect of donor animal species and their feeding on the composition of the microbial community establishing in a rumen simulation. *J Appl Microbiol* 2015;119(1):33-46.
- (50) Firkins JL, Yu Z. Rumen nutrition symposium: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J Anim Sci* 2015;93:1450-1470.
- (51) Kim JN, Henriksen ED, Cann IKO, Mackie RI. Nitrogen utilization and metabolism in *Ruminococcus albus* 8. *Appl Environ Microb* 2014;80(10):3095-3102.
- (52) Cheng KJ, McAllister MA. Compartmentation in the rumen. In: PN Hobson, CS Sewart, editors, *The rumen microbial ecosystem*, Oxford: Blackie Academic & Professional 1997:492-522.
- (53) Chen J, Weimer PJ. Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria. *Microbiology* 2001;147(1):21-30.
- (54) Gorosito AR, Russell JB, VanSoest PJ. Effect of carbon-4 and carbon-5 volatile fatty acids on digestion of plant cell wall *in vitro*. *J Dairy Sci* 1985;68:840-847.
- (55) Jouany J. Effect of rumen protozoa on nitrogen utilization by ruminants. *J Nutr* 1996;126(4 Suppl.).
- (56) Newbold CJ, De la Fuente G, Belanche A, Ramos-Morales E, McEwan NR. The role of ciliate protozoa in the rumen. *Front Microbiol* 2015;6(Nov).
- (57) Williams A, Coleman G. *The Rumen Protozoa*, New York: Springer-Verlag 1992.
- (58) Hino T, Russell JB. Relative contributions of ruminal bacteria and protozoa to the degradation of protein *in vitro*. *J Anim Sci* 1987;64(1):261-270.
- (59) Pilgrim AF, Gray FV, Weller RA, Belling CB. Synthesis of microbial protein from ammonia in the sheeps rumen and the proportion of dietary nitrogen converted into microbial nitrogen. *Brit J Nutr* 1970;24(2):589-598.
- (60) Paster BJ, Russell JB, Yang CMJ, Chow JM, Woese CR, Tanner R. Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp-nov. *Int J Syst Bacteriol* 1993 Jan;43(1):107-110.

- (61) Ricke SC, Martin SA, Nisbet DJ. Ecology, metabolism, and genetics of ruminal selenomonads. *Crit Rev Microbiol* 1996;22(1):27-65.

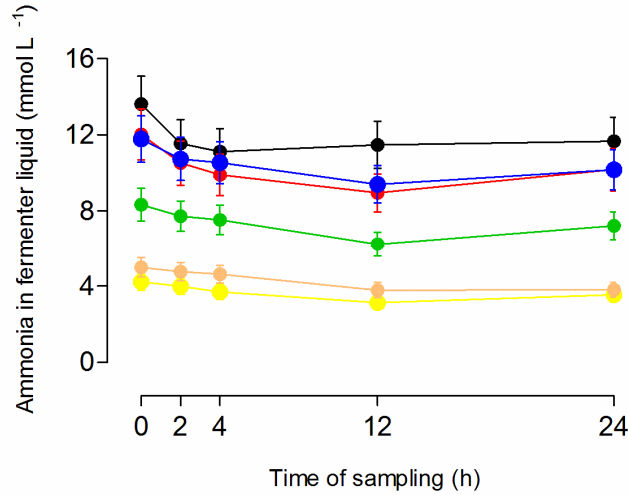


Figure 1: Ammonia concentration in fermenter liquids measured at different sampling times after adding a new feed bag

MS (maize silage, yellow), MS+Urea (black), MS+AA mix (red), MS+Peptone (blue), MS+Protein (orange), GS (grass silage, green; LS means, SD, back-transformed,  $n \geq 4$ ).

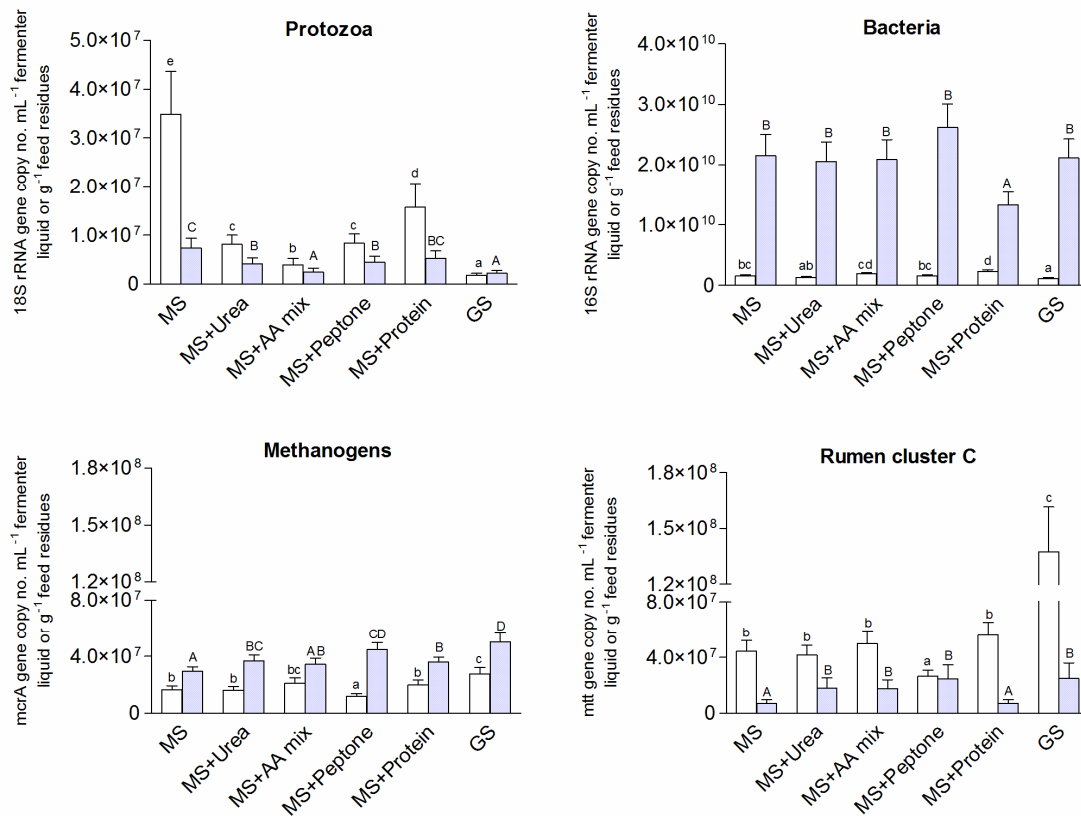


Figure 2: 18S rRNA, 16S rRNA, and mcrA gene copy numbers of protozoa, bacteria, methanogens, and Archaea belonging to rumen cluster C (RCC) detected in fermenter liquid (white) and feed residues (grey)

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed,  $n \geq 4$ ).

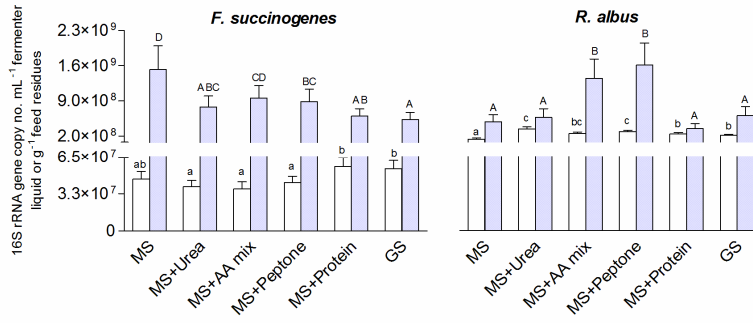


Figure 3: 16S rRNA gene copy numbers of *Fibrobacter succinogenes* and *Ruminococcus albus* in fermenter liquid (white) and feed residues (grey)

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed, n ≥ 3).

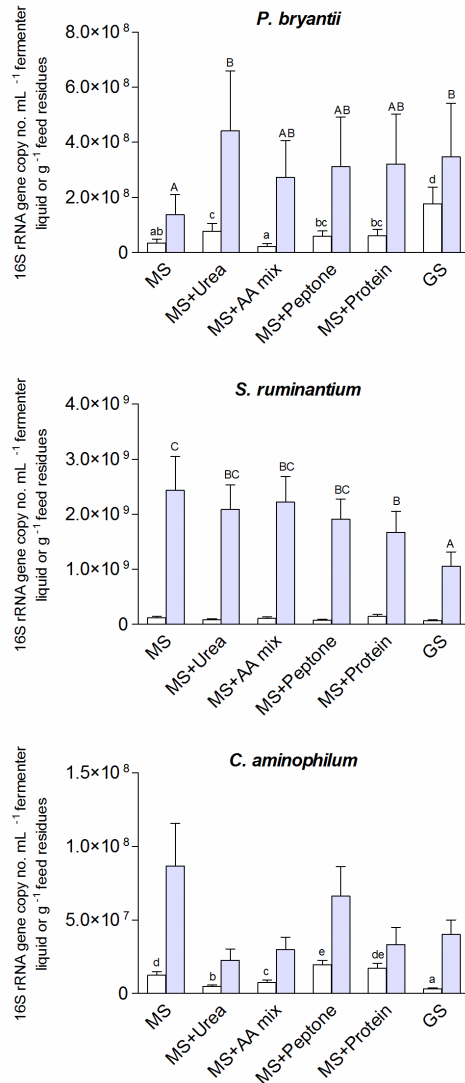


Figure 4: 16S rRNA gene copy numbers of *Prevotella bryantii*, *Selenomonas ruminantium*, and *Clostridium aminophilum* detected in fermenter liquid (white) and feed residues (grey)

MS (maize silage), AA (amino acids) mix, GS (grass silage). Different lowercase and uppercase letters denote significant differences between dietary treatments within fermenter liquids and feed residues, respectively (LS means, SD, back-transformed, n ≥ 4).

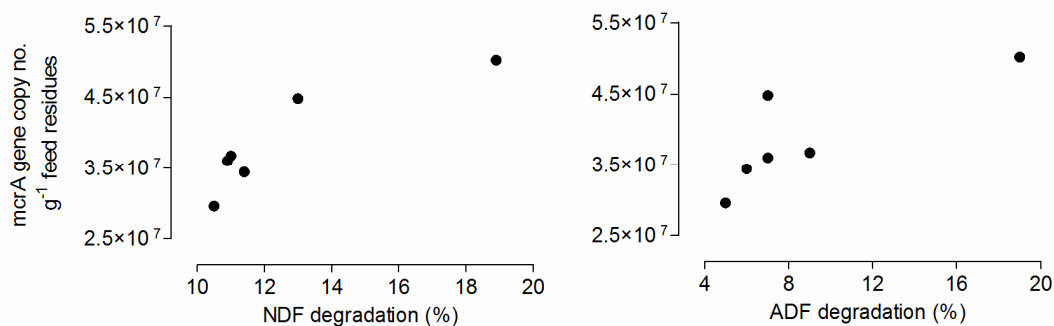


Figure 5: Pearson correlation between total numbers of methanogens in feed residues and degradation of neutral ( $r = 0.89$ ,  $P = 0.017$ ) and acid detergent fibre ( $r = 0.81$ ,  $P = 0.048$ )

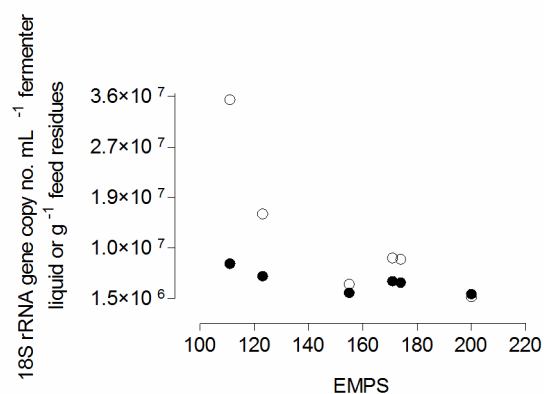


Figure 6: Pearson correlation between protozoal 18S rRNA gene copy numbers in fermenter liquid (open;  $r = -0.85$ ,  $P = 0.034$ ) or feed residues (filled;  $r = -0.82$ ,  $P = 0.048$ ) and the efficiency of microbial protein synthesis (EMPS, g microbial crude protein kg<sup>-1</sup> fermented organic matter)







### MANUSCRIPT 3:

## EFFECTS OF CORN SILAGE AND GRASS SILAGE IN RUMINANT RATIONS ON DIURNAL CHANGES OF MICROBIAL POPULATIONS IN THE RUMEN OF DAIRY COWS

Melanie B. Lengowski<sup>a</sup>, Maren Witzig<sup>a</sup>, Jens Möhring<sup>b</sup>, Gero M. Seyfang<sup>a</sup>, Markus  
Rodehutschord<sup>a</sup>

<sup>a</sup> Universität Hohenheim, Institut für Nutztierwissenschaften, 70599 Stuttgart, Germany;

<sup>b</sup> Universität Hohenheim, Institut für Kulturpflanzenwissenschaften, Fachgebiet Biostatistik,  
70599 Stuttgart, Germany

Published in:

Anaerobe (2016), 42: 6-16

(Doi:10.1016/j.anaerobe.2016.07.004)



## ABSTRACT

The object of this study was to investigate diurnal changes in the ruminal microbial community as well as fermentation characteristics of dairy cows fed total mixed rations containing either corn silage or grass silage as forage source. The rations contained 52% concentrate and 48% grass silage or corn silage and were offered for *ad libitum* intake over 20 days to three ruminal-fistulated lactating Jerseys cows during three consecutive feeding periods. Feed intake, ruminal pH, concentrations of short chain fatty acids and ammonia in rumen as well as changes in numbers of microbial populations in liquid and solid fractions were measured on day 18 and 20 in 4-h intervals. Numbers of total bacteria and *Fibrobacter succinogenes* increased in solid fractions in cows fed corn silage instead of grass silage, and that of protozoa increased in both fractions. Cows fed with grass silage showed higher abundance of *F. succinogenes* and *Selenomonas ruminantium* in the liquid fraction as well as the numbers of *Ruminobacter amylophilus*, *Prevotella bryantii*, *Ruminococcus albus* and *Ruminococcus flavefaciens* in both fractions. Furthermore, effects of silage were detected on the methanogens. The effects of silage which were observed on the ruminal microbial community were not found on the fermentation characteristics since these were less affected by forage sources. Results of this study suggest a functional adaptability of the ruminal microbial community to total mixed rations containing either grass silage or corn silage as forage source. Diurnal changes in ruminal microbial community were primarily affected by feed intake and differed between species and fractions. In the solid fractions the temporal fluctuations were less compared to the liquid fractions. For most of the species which were examined in this study interactions between forage source and sampling time were of major importance. Diurnal changes of microbial populations and fermentative activity were less affected by the forage source.



## CHAPTER 4

### SUMMARY



## 4 SUMMARY

Grass silage and corn silage are the most commonly used silages for feeding dairy cows and fattening bulls. Due to their different chemical composition these silages have diverse effects on the ruminal microbial community and, therefore, on the fermentation process in the rumen. Corn silage, which has a high amount of readily fermentable carbohydrates, promotes amylolytic microorganisms while grass silage, which has a high amount of fiber and protein, promotes cellulolytic and proteolytic microorganisms. The major objective of this thesis was to evaluate the effect of grass silage and corn silage on the ruminal microbial community composition. The focus was on the incubation of silages without using concentrates. Furthermore, diurnal changes of the ruminal microbial community were investigated.

In the first study (Manuscript 1), effects of incubation of grass silage and corn silage on the ruminal microbial community were investigated using an established rumen simulation technique (Rusitec). Furthermore, diurnal changes and changes during the first 48 hours of incubation (adaption phase) were observed. A significant decrease of the complete ruminal microbial community in the fermenter liquids, on species and group level, was observed. During the adaption phase, the silage source lost relevance because in the silage-containing fermenters as well as in the blank fermenters decreasing numbers were observed with the exception of *Clostridium aminophilum*. For this species, after 48 hours higher numbers were found as compared to the inoculum. As has already been described in the literature, in the current thesis changes in microbial abundance were lesser in feed residues than in fermenter liquids. The abundance of protozoa in feed residues decreased during the first 48 hours while for *Prevotella bryantii* and *C. aminophilum* higher numbers were found after 24 and 48 hours as compared to the inoculum. The adaption in the Rusitec had not yet been fully completed after 48 hours, since for several species a different abundance was found on day 13 of incubation (Period 2). The provision of fresh substrate at the beginning of Period 2 led to an increase of almost all species and groups within the following two to four hours, with the exception of the protozoa and methanogens. The reduction of the protozoal population and methanogens could have been the result of migration from fermenter liquids to new feed particles and also the fact that some species of methanogens are associated to protozoa. Likewise, sampling of the feed residues after 24 and 48 hours was too late since the microorganisms could have been already detached. However, sampling of the feed residues at shorter intervals is not possible in the Rusitec. No effect of silage was observed on the abundance of total bacteria, methanogens and *Selenomonas ruminantium*. Methanogens and *S. ruminantium* do not use the substrate itself but rather fermentation end products of other ruminal microorganisms. Although no effect of silage was observed on the abundance of methanogens, more methane was produced during the incubation of grass silage than corn silage. This is in accordance with results described in literature that an abundance of methanogens is not associated

with the production of methane. The corn silage promoted the abundance of protozoa and *Ruminobacter aminophilus* whereby the latter could only be detected during the first 48 hours of incubation. Quantification was not possible because of the generation of unspecific products during real-time qPCR. *Fibrobacter succinogenes* was also promoted by corn silage even though this is one of the most active cellulolytic species in the rumen. It could be possible that this species had an advantage in the degradation of the cell wall structures of C<sub>4</sub> plants (which corn accounts to) as compared to other cellulolytic species. The grass silage promotes the abundance of the cellulolytic *Ruminococcus albus* as well as *P. bryantii* and *C. aminophilum*. The last two species use amino acids and proteins as energy sources thereby the latter belongs to hyper ammonia producing bacteria which could be the reason for the higher ammonia concentration in fermenter liquids during the incubation of grass silage compared to corn silage.

In the second study (Manuscript 2), the effects of supplementing corn silage with different nitrogen sources on ruminal microbial community composition and ruminal microbial crude protein synthesis was investigated. Higher efficiency of microbial crude protein synthesis with corn silage has been reported from *in vivo* studies, while *in vitro* contradictory results were found. It was proposed that the different nitrogen content of the silages, and especially the lack of rumino-hepatic circulation *in vitro*, could be the reason for the lower efficiency of microbial crude protein synthesis when using corn silage. Different microbial species prefer different nitrogen sources as already observed in pure cultures and partially also in mixed cultures. Thus, in the second study, corn silage was supplemented with urea, pea protein, pea peptone or mixed free amino acids. With the supplementation of peptone and urea to corn silage, the highest efficiency of microbial crude protein synthesis was observed followed by amino acids and protein supplementation. But none of the used nitrogen sources allowed corn silage to achieve the level of microbial crude protein synthesis observed for grass silage. The protozoal population was negatively correlated with the efficiency of microbial crude protein synthesis which could have been a result of the higher energy requirement of protozoa compared to bacteria. Furthermore, protozoa engulfs and ingests bacteria and uses it as the main energy and protein source which could also have a negative effect on the efficiency of microbial crude protein synthesis. The effects of different nitrogen sources on the abundance of microbial groups and species not always were in accordance with reports in the literature. This could be attributed to the use of different substrate and pure cultures.

In the third study (Manuscript 3), the effect of grass silage and corn silage on the ruminal microbial community composition *in vivo* was investigated. Three lactating Jersey cows fitted with a permanent cannula were fed with rations based on grass silage or corn silage. The rations contained the same amount of concentrate. In order to adjust the nitrogen content of the rations the corn silage based ration was supplemented with urea. The corn silage based ration promoted



the abundance of total bacteria in the solid fraction obtained from the rumen, as well as the protozoal population in the solid and liquid fraction. It could be possible that the higher content of readily fermentable carbohydrates, mainly starch, of the corn silage in combination with the urea could have had a positive effect on both groups. Furthermore, a higher abundance of *F. succinogenes* was observed in animals fed with the corn silage-based ration. As expected, the cellulolytic bacteria *R. albus* and *R. flavefaciens* were enhanced by the grass silage-based ration as well as *P. bryantii* and *R. amylophilus*. The latter are known to use starch and maltose as energy source and to use their proteolytic activity only to achieve access to protein-coated starch. It is assumed that the combination of grass silage and concentrate could have had an influence on this species. For *S. ruminantium*, a feeding effect on the abundance was only observed in the liquid fraction while in the solid fraction no effect was found. There was also no observable effect of ration on the abundance of *C. aminophilum*, RCC and the methanogens. Changes of the ruminal microbial community over time were observed. But no consistent increase of all species and groups after the phase with the highest dry matter intake of fresh feed became obvious.

In conclusion, the results of the current thesis showed that grass and corn silages differently affected the composition of the ruminal microbial community in the liquid and solid fractions of the rumen content. Furthermore, significant diurnal changes of the ruminal microbial community could be observed *in vitro* as well as *in vivo*. These results highlighted the need of repeated sampling during the day and suggest the consideration of both ruminal compartments, liquid and solid.



## CHAPTER 5

### ZUSAMMENFASSUNG



## 5 ZUSAMMENFASSUNG

In Deutschland und Europa zählen Gras- und Maissilage zu den am häufigsten eingesetzten Silagen in der Fütterung von Milchkühen und Mastrindern. Aufgrund deren unterschiedlicher chemischer Zusammensetzung zeigen sich unterschiedliche Auswirkungen auf die Mikroorganismengemeinschaft und somit den Fermentierungsprozess im Pansen. Die Maissilage, mit ihrem hohen Anteil an leicht verdaulichen Kohlenhydraten, fördert die Bildung von amylolytischen Mikroorganismen, während die Grassilage, mit ihrem hohen Anteil an Fasern (NDF und ADF), sowie dem hohen Proteingehalt, die cellulolytischen und proteolytischen Mikroorganismen fördert. Hauptziel dieser Arbeit war es daher, den Einfluss der unterschiedlichen Silagen auf die ruminale mikrobielle Gemeinschaft zu untersuchen. Der Schwerpunkt lag dabei auf der Beurteilung des alleinigen Effekts der Silagen, ohne Zulage von Kraftfutter. Zusätzlich wurde die zeitliche Veränderung der Mikroorganismengemeinschaft untersucht.

In der ersten Studie (Manuskript 1) wurden der Effekt der alleinigen Inkubation von Gras- und Maissilage auf die ruminale mikrobielle Gemeinschaft im Rusitec untersucht, sowie die zeitliche Veränderung und die Adaptionsphase während der ersten 48 h. Dabei konnte eine deutliche Verringerung der gesamten mikrobiellen Gemeinschaft in der Fermenterflüssigkeit, sowohl auf Spezies- als auch auf Gruppenebene beobachtet werden. Die Silagen spielten in dieser Zeit nur eine untergeordnete Rolle, da kein Unterschied zwischen den Blindwert-Fermentern und den Silage-Fermentern festgestellt wurde. Eine Ausnahme war für *Clostridium aminophilum* zu beobachten, für den nach 48 h eine höhere Konzentration als im Inokulum nachgewiesen wurde. Wie bereits in der Literatur beschrieben, konnten auch in dieser Studie insgesamt geringere Konzentrationsveränderungen in den Beutelnrückständen im Vergleich zur Fermenterflüssigkeit beobachtet werden. So zeigten die Protozoen in den Beutelnrückständen während der Adaptionsphase eine deutliche Reduktion, währenddessen für *Prevotella bryantii* und *C. aminophilum* eine deutlich höhere Anzahl nach 24 h und 48 h zu beobachten war. Nach 48 h schien die Adaptionsphase noch nicht vollständig abgeschlossen zu sein, da zu Beginn der 2. Periode (Tag 13) für einige Spezies unterschiedliche Konzentrationen nachweisbar waren. Nach der Inkubation von frischem Substrat (in Periode 2) war ein deutlicher Anstieg der Abundanz aller untersuchten Spezies und Gruppen, mit Ausnahme der Protozoen und Methanogenen, innerhalb der ersten zwei bis vier Stunden in der Fermenterflüssigkeit beobachtbar. Die Reduktion der Protozoen und Methanogenen ist wohl auf eine aktive Abwanderung der Protozoen aus der Fermenterflüssigkeit hin zum neuen Substrat zurückzuführen und die Assoziation einiger Methanogenen an die Protozoen. Es ist anzunehmen, dass die Untersuchung der Beutelnrückstände nach 24 h und 48 h suboptimal ist, da sich zu diesem Zeitpunkt die Mikroorganismen bereits wieder abgelöst haben könnten. So konnten kaum Veränderungen der partikel-assoziierten

Mikroorganismen nachgewiesen werden. Allerdings ist eine Probenahme aus den Beutelnrückständen in einem kürzeren Zeitintervall im Rusitec nicht umsetzbar. Es konnte kein Effekt der Silagen auf die Gesamtbakterien, sowie die Methanogenen und *S. ruminantium*, beobachtet werden. Die Methanogenen, als auch *S. ruminantium*, nutzen nicht direkt das Substrat, sondern Fermentationszwischen- und endprodukte anderer Mikroorganismen. Obwohl keine Veränderung in der Anzahl der Methanogenen zu beobachten war, wurde während der Inkubation von Grassilage mehr Methan gebildet. Dies deckt sich mit Ergebnissen aus der Literatur, in denen kein Zusammenhang zwischen der Anzahl der Methanogenen und der Methanbildung festgestellt werden konnte. Die Maissilage förderte die Protozoen und *R. amylophilus*, wobei letzterer nur in den ersten 48 h quantifiziert werden konnte. Anschließend bildeten sich während der real-time qPCR unspezifischen Produkte, die eine eindeutige Quantifizierung nicht zuließen. Ebenso war die Anzahl von *F. succinogenes*, einer der aktivsten Zellulolyten, bei der Inkubation von Maissilage höher. Es ist anzunehmen, dass diese Spezies im Vergleich zu anderen zellulolytischen Spezies einen Vorteil beim Abbau von Zellwänden von C<sub>4</sub>-Pflanzen, zu denen die Maispflanzen gehören, hat. Die Grassilage förderte den Zellulolyten, *Ruminococcus albus* sowie *P. bryantii* und *C. aminophilum*. Die beiden letzt genannten nutzen Aminosäuren und Peptide als Energiequelle, wobei letzterer zu den hyper ammonia producing bacteria (HAB) zählt. Dies könnte der Grund für die höhere Ammoniakkonzentration in der Fermenterflüssigkeit während der Inkubation von Grassilage sein.

Aufgrund von vorangegangenen Studien wurde in der zweiten Studie (Manuskript 2) der Effekt von unterschiedlichen Stickstoffquellen auf die Mikroorganismen und die Effizienz der mikrobiellen Proteinsynthese untersucht. *In vivo* konnte im Vergleich zur Maissilage bei der Verfütterung von Grassilage eine höhere Effizienz der mikrobiellen Proteinsynthese beobachtet werden. *In vitro* wurden hingegen gegenläufige Ergebnisse gefunden. Nach ersten Recherchen war davon ausgegangen worden, dass der unterschiedliche Stickstoffgehalt der Silagen und vor allem der fehlende ruminohepatische Kreislauf *in vitro*, Grund für die verminderte Effizienz der mikrobiellen Proteinsynthese war. Allerdings konnte nach Zulage von Harnstoff zur Maissilage kein zufriedenstellendes Ergebnis erzielt werden. Wie bereits in Reinkulturen und zum Teil in Mischinkubationen nachgewiesen, bevorzugen unterschiedliche Spezies unterschiedliche Stickstoffquellen. Somit wurde in der zweiten Studie Maissilage entweder mit Harnstoff, Erbsenprotein, Erbsenpepton oder einer Mischung aus freien Aminosäuren ergänzt. Durch die Zulage von Pepton und Harnstoff konnte innerhalb der Stickstoffzulagen die höchste Effizienz der mikrobiellen Proteinsynthese erzielt werden. Gefolgt von den Aminosäuren und dem Protein. Allerdings konnte keine Variante das Niveau der Grassilage erreichen. Die Protozoen waren negativ mit der Effizienz der mikrobiellen Proteinsynthese korreliert, was auf den erhöhten Energiebedarf der Protozoen zurückgeführt werden könnte. Außerdem nutzen Protozoen

Bakterien als Energie- und Proteinquelle, was ebenfalls einen negativen Effekt auf die Effizienz der mikrobiellen Proteinsynthese haben dürfte. Die Effekte der Stickstoffzulage auf die Abundanz einzelner Spezies und Gruppen stimmten nicht immer mit Berichten in der Literatur überein. Dies kann zum Teil auf die Verwendung von Reinkulturen und unterschiedlichen Substraten zurückgeführt werden.

In der dritten Studie (Manuskript 3) wurde der Effekt von Mais- und Grassilage auf die ruminale mikrobielle Gemeinschaft *in vivo* untersucht. Dazu wurde drei Jersey-Rindern entweder eine Grassilage oder Maissilage basierte Ration mit dem gleichen Anteil Kraftfutter verfüttert. Zum Ausgleich des höheren Stickstoffgehaltes der Grassilage wurde der Maissilage Harnstoff hinzugefügt. Die Maissilage basierte Ration förderte die Gesamtbakterienzahl in der festen Phase, sowie die Protozoen in beiden Phasen. Es könnte möglich sein, dass der höhere Gehalt an leicht verdaulichen Kohlenhydraten, die in der Maissilage vermehrt vorkommen, in Kombination mit dem Stickstoff aus der Harnstoffzulage diese beiden Gruppen förderte. Ebenfalls war wieder eine höhere Abundanz von *F. succinogenes* in der festen Phase bei der Fütterung der Maissilage-basierten Ration zu beobachten. Wie erwartet, wurden die Zellulolyten *R. albus* und *Ruminococcus flavefaciens* durch die Fütterung der Grassilage-basierten Ration gefördert. Ebenso wie *P. bryantii* und *R. amylophilus*, wobei letzterer Stärke und Maltose als Energiequelle nutzt und seine proteolytische Aktivität nur benötigt, um Proteinummantelte Stärkekörner freizulegen. Es ist anzunehmen, dass die Kombination aus Grassilage und Kraftfutter einen positiven Einfluss auf diese Spezies hatte. *S. ruminantium* war nur in der flüssigen Phase durch die Fütterung der Grassilage basierten Ration gefördert worden, während in der festen Phase kein Effekt der Ration nachgewiesen werden konnte. Ebenso konnte kein Effekt der Ration auf *C. aminophilum*, Rumen Cluster C (RCC) und die Methanogene beobachtet werden. Wie bereits *in vitro*, konnte auch *in vivo* eine deutliche Veränderung der mikrobiellen Gemeinschaft über die Zeit nachgewiesen werden. Allerdings war kein einheitlicher Anstieg aller Spezies, nach der Aufnahme einer größeren Menge Futters, wie *in vitro* der Fall war, zu beobachten gewesen.

Die Ergebnisse der vorliegenden Arbeit zeigen, dass unterschiedliche Silagen zu einer Veränderung in der Zusammensetzung der ruminalen mikrobiellen Gemeinschaft führen. Desweiteren konnte eine deutliche Veränderung der mikrobiellen Gemeinschaft innerhalb eines Tages nachgewiesen werden. Dies unterstreicht die Notwendigkeit, einer wiederholten Probenahme, um eine repräsentative Aussage über den Effekt auf die mikrobielle Gemeinschaft treffen zu können.





## ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to Prof. Dr. Markus Rodehutschord, for his excellent guidance through all stages of this work. His constructive criticism and discussions over the last few years have been invaluable. I am grateful for his trust he placed in me, the possibility to implement my own ideas in the experiments, as well as for training me in scientific work.

I offer special thanks to Dr. Maren Witzig for her encouragement through all stages of this work and her untiring support during analysis of samples in the laboratory. Armed with immense knowledge, she has always been by my side with helpful proposals. I also thank her for the excellent assistance during the writing process.

I am particularly grateful for the assistance given by Beate Mezger-Henschel during analysis in the laboratory. I will never forget our delightful times together. I am also very grateful to Melanie Berger for analyzing my last samples.

I also thank Karin Zuber and the members of the Department of Animal Nutrition for their support during the Rusitec-experiments. I would also like to express thanks to Gero Seyfang, for allowing me to participate in his *in vivo* experiment, and PD Dr. Jeannette Boguhn for her help at the start of my work. Thanks to the members of the Department of Soil Biology for giving me the opportunity to use their NanoDrop for spectrophotometric measurements of samples.

My sincere thanks also go to Jens Möhring for his active assistance in all matters of statistics, and his enormous patience with all my questions.

I am also grateful to all my PhD fellows, for the fun and sport of the last four years. It was a great time! Special thanks to Jochen Krieg, who, sometimes, had to endure my moods.

My deepest and heartier thanks go to my family and Philipp Hofacker, who have supported me over the years. All of them were fundamental in supporting me during the last four years. Without them, this doctorate would have been impossible. I am truly thankful to the possibility to thank my mother personally for her support over the last years. I also thank my friend Jacqueline Schmolz, who spends a lot of her weekends and holidays with my laptop and me.



# CURRICULUM VITAE

## PERSONAL DATA

---

Name: Melanie Lengowski  
Date of birth: 25.04.1987  
Place of birth: Leonberg

## EDUCATION

---

Since 11/2015 Product manager; Timac Agro Deutschland GmbH, Troisdorf, Germany  
7/2012 – 5/2015 Research for PhD, Department of Animal Nutrition, University of Hohenheim, Stuttgart, Germany  
4/2010 – 6/2012 Master Study in Agricultural Science (Animal Nutrition), University of Hohenheim, Stuttgart, Germany  
Degree: M.Sc.  
10/2006 – 3/2010 Bachelor Study in Agriculture, HfWU Nürtingen-Geislingen, Nürtingen, Germany  
Degree: B.Sc.  
9/2004 – 4/2006 Vocational school for Agricultural- and Environmental analysis (livestock farming), Stuttgart-Hohenheim, Germany  
Qualification gained:

- state-certified technical assistant for Agricultural- and Environmental analysis
- advanced technical certificate

## PRACTICAL EXPERIENCE

---

2/2012 - 3/2012 Student Assistant at Department of Animal Nutrition, University Hohenheim, Stuttgart, Germany  
10/2006 – 07/2012 Part-time job at Krämer Pferdesport, Heimsheim, Germany  
4/2010 and 9/2010 - 10/2010 Part-time job at Spedition Keller Logistik, Ditzingen, Germany  
11/2008 – 12/2008 Internship on a dairy farm, Christchurch, New Zealand  
8/2007 – 9/2007 Internship at Rinderunion Baden Württemberg e.V., Herbertingen, Germany

Bräunlingen, 22nd of September 2016

---

Melanie Lengowski



## **EIDESSTATTLICHE VERSICHERUNG**

Bei der von mir eingereichten Dissertation zum Thema „Studies on the composition of the ruminal microbial community using grass silage and corn silage“ handelt es sich um meine eigenständig erbrachte Leistung. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erkläre und nichts verschwiegen habe.

Bräunlingen, den 22. September 2016

---

Melanie Lengowski





